Development and Use of a DNA Microarray for the Detection of Enteritic Pathogens in Cattle and Pigs

Aouatif Belkhiri Dipl. Ing., MSc

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Abstract

Enteritis is a very frequent cause of morbidity and mortality in young calves and pigs. They may be infected with 54 known pathogens, particularly in the first months of their life. Simultaneous infection with multiple pathogens occurs frequently and produces a synergistic effect in terms of the severity of clinical disease. In this study two microarray platforms (Agilent and Alere) were used to detect enteric pathogens. A total of 15993 probes were designed from viral, bacterial and parasitic sequences using four different software (UPS, Picky, eArray and GoArray). The probes for the Alere platform were assessed thermodynamically individually for secondary structure formation and hybridised to their complementary sequences *in silico*. Specificity and sensitivity testing was done with reference strains and porcine and bovine clinical samples and were performed with both platforms.

The Alere ArrayTube platform holding 201 probes was used to identify viruses in reference and clinical samples. Among eight reference virus strains, five (PEDV, TGEV, PCV-2, BVDV and PPV) were identified correctly and of these PEDV, TGEV, PCV-2 were confirmed by PCR using designed primers Two viruses, P. rotavirus A and P. bocavirus were negative by array but were confirmed by PCR, rotavirus by designed primers and bocavirus by published primers. In two hybridisations using multiplex PCR products from two separate sets each of 5 mixed pathogens, the ArrayTube detected all viruses for one set and only one virus out of two in the other set. The specificity test using three non-enteric

viruses showed high background noise for Bunyamwera virus and Schmallenberg virus, however only one probe cross-hybridised with Equine influenza virus. The sensitivity of this platform showed that it can detect an amount of 2.065 x 10^9 copy number (2 ng) of PCV-2 and 2.420 x 10^5 copy number (39 pg) of TGEV present in the sample.

The results of hybridised reference viral strains and clinical samples showed that random amplification was more favourable for reference strain detection compared to specific amplification. However, specific amplification performed better for clinical samples.

The Agilent microarray platform, comprising 44000 probes of enteric bacteria, viruses and parasites, was subjected to hybridisation of 12 reference strains for specificity testing (four viruses, seven bacteria and one parasite). All hybridised strains were correctly detected except P. rotavirus A which showed only 7 positive probes, however with high signal intensities. A high level of cross-hybridisation was observed with this platform due to the 16S rRNA and 18S rRNA probes as these two genes were amplified in their entirety prior to hybridisation and a high degree of similarity exists between of 16S rRNA and 18S rRNA among different strains of bacteria and parasites respectively.

Hybridisation of PCR products to the Agilent platform from two sets of five multiplexed pathogens showed that all ten pathogens were correctly identified. The sensitivity results of this platform showed that it can detect 2.065 x 10^9 copy number of PCV-2, equivalent to a viral load of 2 ng. On the other hand the detection limit of *E. coli* F5 was comparable to the real-time PCR technique with

a minimum of 2.089 x 10^8 copy number of *E. coli* fimbrial gene present in the sample.

In bovine clinical samples, the Agilent microarray was able to identify the presence of *E. coli* F5 in two samples out of four tested. However, in porcine clinical samples, the array successfully detected all pathogens whose presence was confirmed by PCR. Mixed infections in porcine samples were also detected by microarray, where *Clostridium difficile* with its toxins (toxin A *tcdA* and binary toxin *cdt*), P. rotavirus and P. kobuvirus were detected simultaneously in one sample. It also detected the presence of the *C. difficile* clindamycin resistance gene (*ermF*) in another sample.

In this study microarray technology has been shown to have the potential to detect mixtures of enteric pathogens in bovine and porcine faecal samples. It also has genotyping abilities for exploration of genetic variation. However, the sensitivity and specificity could be improved with more *in silico* assessments of designed probes. Eventually testing with a higher number of reference strains and clinical samples is necessary.

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Dedication

A mon très cher Papa, qui nous a quitté le Mercredi 20 Juillet 2016. Repose en paix.

To my Dear Dad, who passed away on the 20th of July 2016. Rest in peace.

Fifette

Declaration

Unless otherwise acknowledged, the work presented in this thesis is original. No part has been submitted for another degree at the University of Nottingham or elsewhere. Any views expressed in the dissertation are those of the author.

Signed ……..

Date ……..

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List of Abbrevations

A	Adenine
ANOVA	Analysis of Variance
AT	ArrayTube
AUD	Australian Dollar
BC	Base Composition
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool nucleotide
Bp	Basepair
BSA	Bovine Serum Albumin
BVDV	Bovine Viral Diarrhoea Virus
BVDVcp	Cytopathic Bovine Viral Diarrhoea Virus
BVDVncp	Non-cytopathic Bovine Viral Diarrhoea Virus
C	Cytosine
cal/K/mol	calories per Kilo per mole
cDNA	Complementary DNA
CFU	Colony Forming Unit
Cm2	Square centimetre
CPV	Canine parvovirus
Cy	Cyanine
dATP	Deoxyadenisine triphosphates
dCTP	Deoxycytidine triphosphates

dGTP	Deoxyguanosine triphosphates
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphates
ds	Double stranded
dsRNA	Double stranded RNA
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphates
dUTP	Deoxyuridine triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
F	Forward
FASTA	FAST-All (Rapid heuristic search method for protein or nucleotide sequence data).
FPV	Feline Panleukopenia Virus
g	Gravity
G	Guanine
Gb	Gigabase
GB	Gigabyte
GC	Guanine-Cytosine
GE	Gene Expression
GHz	Gigahertz
h	Hour
Hg	Inches Mercury
HPLC	High Performance Liquid Chromatography

ICTV	International Committee on Taxonomy of Viruses
ITS	Intergenic Spacer
Kb	Kilobase
KCal/mol	KiloCalories per mole
kDa	KiloDalton
LAMP	Loop-Mediated Isothermal Amplification
LPS	Lipopolysaccharide
M	Million
M	Molar
MFEprimer	Multiple Factor Evaluation of the specificity of PCR primers.
mg	Milligram
Mg	Magnesium
MgCl2	Magnesium Chloride
min	Minute
ml	Millilitre
MM	Mismatch
mM/mmol/L	Millimolar
mRNA	Messenger RNA
MVM	Minute Virus of Mice
NCBI	National Centre of Biotechnology Information
ng	Nanogram
NGS	Next Generation Sequencing
nm	Nanometre

nm	Nanometre
nr	Non Redundant
NS	Non Structural
NSP	Non Structural Protein
nt/NT	Nucleotide
NTPase	RNA Nucleoside Triphosphatase
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PCV	Porcine Circovirus
PEDV	Porcine Epidemic Diarrhoea Virus
pg	Picogram
PM	Perfect Match
PMWS	Post- weaning Multi-systemic Wasting Syndrome
PRCV	Porcine Respiratory Coronavirus
qPCR	Quantitative Polymerase Chain Reaction
R	Reverse
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic Acid
rpm	Rotation per minute
RT	Real Time
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification

RTP	Ready To Prep
RT-PCR	Real Time Polymerase Chain Reaction
SAM	Sentrix Array Matrix
SDS	Sodium Dodecyl Sulfate
sec	Second
SEM	Standard Error of Mean
SPF	Specific Pathogen Free
SPI	Salmonella Pathogenicity Island
SPSS	Statistical Package for the Social Sciences
ss	Single stranded
SSC	Saline Sodium Citrate
ssDNA	Single stranded DNA
ssRNA	Single stranded RNA
T	Thymine
TARMA	Tetramethylrhodamine
TB	Terabyte
TGEV	Transmissible Gastroenteritis Virus
Tm	Melting Temperature
TMB	Tetramethylbenzidine
U/µl	Unit/Microliter
UK	United Kingdom
UPS	Unique Probe Selector
US	United States

USA	United States of America
USDA	United States Department of Agriculture
UTR	Untranslated Region
v/v	Volume/Volume
VP	Virulence Plasmid
VP	Virus Protein
WGS	Whole Genome Sequencing
ΔG/DeltaG	Free Energy
ΔH	Enthalpy
ΔS	Entropy
°C	Degree Celsius
µl	Microliter
µm	Micrometre
µM	Micromolar
16S rRNA	16S ribosomal RNA (small subunit)
23S rRNA	23S ribosomal RNA (large subunit)
6-FAM	6- Carboxyfluorescein

Chapter 1. Introduction and Literature Review

1. 1. Infectious enteritis

Enteric infections (enteritis) involving young animals are a major cause of economic loss in most countries where cattle and pig rearing are an important component of the livestock industry. Enteritis is generally characterised by diarrhoea which is a consequence of hypersecretion and malabsorption, the occurrence of one or both phenomena leading to dehydration in the acute form of the disease. Hypersecretion is due to the loss of electrolytes and fluids (Lorenz et al., 2011) resulting from released toxins by enteropathogens (Hodges and Gill, 2010). However, malabsorption is the result of functional and anatomical atrophy or loss of the absorptive cells of the intestine as a result of the infection (Crouch, 1985; Crouch and Woode, 1978; Pearson et al., 1978).

1. 2. Economic importance of enteric infections in pig and cattle industry

The costs of enteric infections include decreased production, fees covering veterinary services which include care, treatment, prevention and introduction of control measures. In general, enteric infections are estimated to result in a loss of < 5% of the economic value of the animal (S. McOrist, personal communication to P. Barrow).

Detailed data on economic losses are limited and there are very few studies available which describe the economic impact of enteritis due to a unique specific pathogen. Thus for instance, the total annual loss in the US dairy herds

due to Bovine Viral Diarrhoea virus (BVDV) was estimated at $57 million per million calvings (Houe, 1999). In Canada the estimated financial-economic effects of this pathogen ranged between 240 € and 600 € per cow (Carman et al., 1998), while other reports have indicated a cost of 361 million to $1.4 billion for 2008 in USA (Ridpath et al., 2006; Peña, 2010). In the swine industry, losses related to Transmissible Gastroenteritis virus (TGEV) were reported in the US and estimated to be $200 million per year (Saif and Wesley, 1992). A more recent study conducted in Australia estimated the economic impact of enteritis due to *Lawsonia intracellularis* in pigs as a reduction in the profit by $13AUD per clinically infected pig compared to net revenue of $25.31 AUD in non-infected animals (Holyoake et al., 2010).

Various studies have reported a high mortality rate in calves and piglets due to intestinal infection (Bellows et al., 1987; García- Sánchez et al., 1993; Virtala et al., 1996; Dewey et al., 2006; Mee J.F., 2008; Poljak et al., 2010). In US dairy production, The National Animal Health Monitoring System reported a mortality of 57% in weaning calves (USDA, 2007) and recently, a comparable rate (53.4%) was also observed in dairy calves in Korea (Hur et al., 2013). During the first month after birth calves are at high risk of death due to diarrhoea which diminishes after that period but some risk nevertheless remains until six months of age (Gulliksen et al., 2009).

Mortality and morbidity might be increased when more than one enteropathogen are present (Blanchard, 2012). Enteritis is a complex infection and involves predisposing factors, especially sanitary management, and the

presence of different types of microorganisms such as viruses, bacteria and protozoa (Alfieri et al., 2004).

A large variety of viral, bacterial and parasitic enteropathogens infect the intestinal tract of cattle and pigs populations especially during the first months of life. In this review, detection rates and prevalences from national surveys are reported. It is also clearly stated when the rate of infection is indicated in a meta-analysis study.

1. 3. Pathogens involved in enteritis

1. 3. 1. Viruses

According to the literature, several species of viruses belonging to distinct families are implicated in intestinal infections, and include species/serotypes of the *Caliciviridae*, *Circoviridae, Coronaviridae*, *Picornaviridae*, *Parvoviridae* and *Flaviviridae* familes.

1. 3. 1. 1. *Caliciviridae*

The *Caliciviridae* family comprises non-enveloped single-stranded positive sense RNA viruses of 27-38 nm in diameter and a genome size of 7.3-8.3 kb (Green et al., 2001). The most important genera are *Norovirus*, *Sapovirus* and *Nebovirus* (Saif et al., 1980; Bridger, 1990; Guo et al., 2001; Wang et al., 2007; Hassine-Zaafrane et al., 2012).

a. Norovirus

Noroviruses (NoVs) are important in acute gastroenteritis in young children (Noel et al., 1999) and have also been associated with diarrhoea in calves

(Bank-Wolf et al., 2010; Scipioni et al., 2008) and young cattle (Mauroy et al., 2009a). The role of these viruses in porcine diarrhoea is still unknown (Scipioni et al., 2008). In experiments with gnotobiotic pigs, mild diarrhoea appeared after inoculation (Wang et al., 2005b).

Human strains are genetically related to porcine strains and recombinant NoVs have already been characterised (Farkas et al., 2005; Wang et al., 2005b). Three open reading frames (ORFs) comprise the noroviral genome (Jiang et al., 1993). The non-structural polyprotein, including NTPase, protease and RNA-dependent RNA polymerase (RdRp), is encoded by ORF1 (Sosnovtsev et al., 2006). ORF2 encodes the major capsid protein (VP1), and ORF3 encodes a minor structural protein (VP2) (Belliot et al., 2003).

Noroviruses are highly diverse genetically comprising 31 genotypes within 5 genogroups (Wang et al., 2005b; Zheng et al., 2006) with two additional more recently identified genogroups VI and VII (Pham et al., 2007). Genogroups I, II, IV, VI and VII contain human viruses, of which genogroups I, IV, VI, and VII were detected exclusively in humans (Phan et al., 2007). Genogroup I comprises the Norwalk strain and other human strains whereas genogroup II contains porcine strains, 11, 18 and 19 (Wang et al., 2005a). Bovine noroviruses form their own genogroup III which is divided into two genotypes, Newbury 2 virus (Woode and Bridger, 1978) and Jena virus (Günther et al., 1984; Scipioni et al., 2008).

b. Porcine Sapovirus

Sapovirus (SaV) is one of the emerging pathogens causing diarrhoea in humans, especially children and the elderly and also in animals such as swine and mink

and have a worldwide distribution (Flynn et al., 1988; Green and Chanock, 2001; Guo et al., 2001a, 2001b; Mayo, 2002). A prevalence study on porcine SaVs showed that the GIII/Cowden-like SaVs were the most prevalent strains (Wang et al., 2006). They may be detected in pigs of all ages.

SaVs are genetically variable and have been classified into nine genotypes in five genogroups based on the complete capsid sequences (Schuffenecker et al., 2001; Farkas et al., 2004). Genogroups I, II, IV and V contain human strains and genogroup III comprises animal strains (Farkas et al., 2004). However, several pig strains have been suggested as members of new genogroups VI and VII (Wang et al., 2005a; Martella et al., 2008a). Two other genogroups IX and X were also discovered recently in swine herds (Reuter et al., 2012).

c. Nebovirus

Although viruses related to Nebovirus were detected in the late 1970s in calves in the UK and USA (Woode and Bridger, 1978; Smiley et al., 2002), it is a relatively newly recognized bovine enteric calicivirus (Carstens, 2010). Neboviruses have been isolated from many countries including the USA (Smiley et al., 2003), the UK (Oliver et al., 2006), South Korea (Park et al., 2008), France (Kaplon et al., 2011) and Tunisia (Hassine-Zaafrane et al., 2012) with prevalence rates varying between 3% in Tunisia (Hassine-Zaafrane et al., 2012), where 169 faecal samples were screened during four years time in the central east region of the country, 28% in the USA from a study targeting two farms and 358 faecal samples (Smiley et al., 2003).

The genome contains two ORFs; ORF1 encodes a polyprotein that produces non-structural proteins and the capsid protein (Oliver et al., 2006) while ORF2 encodes a small basic protein with an unknown function (Smiley et al., 2002; Oliver et al., 2006).

1. 3. 1. 2. *Circoviridae*

Porcine circovirus belongs to the family of *Circoviridae*, which is composed of two genera *Circovirus* and *Gyrovirus* (McNulty et al., 2000). The genus *Circovirus* includes Porcine circovirus type 1 (PCV1) and type 2 (PCV2) (Mankertz et al., 1997; Hamel et al., 1998), and a variety of avian circoviruses (Todd, 2004). *Gyrovirus* contains Chicken Anaemia Virus (CAV) (Pearson et al., 1978; Todd et al., 1991).

Porcine circoviruses (PCVs) show a worldwide distribution (Cheung et al., 2007; Segalés et al., 2008) and the infection is ubiquitous in domestic pigs (Allan and Ellis, 2000) with sometimes high prevalence rates, including 99% in the Republic of Korea (Chae and Choi, 2010) and 88% in Australia (Finlaison et al., 2007).

Porcine circoviruses are small non-enveloped ssDNA viruses (Tischer et al., 1974; Todd et al., 2005). They consist of PCV1 and PCV2 belonging to two different genotypes (Meehan et al., 1998). PCV1 is non-virulent (Tischer et al., 1986) whereas PCV2 is considered to be the aetiological agent of Post-weaning Multi-systemic Wasting Syndrome (PMWS) (Allan et al., 1998; Ellis et al., 1998). The two genotypes share about 75% nucleotide sequence identity (Hamel et al., 1998) and both possess 11 predicted ORFs. ORF1 and ORF2, oriented in

opposite directions, are the two major ORFs in both PCV1 and PCV2 (He et al., 2012).

PCV2 can be divided into several groups (Olvera et al., 2007) with two phylogenetic groups, PCV2a and PCV2b, present in pig population (Cheung et al., 2007; Segalés et al., 2008).

Of particular interest is the genetic variation of PCV2 that has been described with a genetic shift from genotype PCV2a to PCV2b, seen in PMWS outbreaks in USA, Canada and Europe in 2007 and 2008 (Cheung et al., 2007; Gagnon et al., 2007; Dupont et al., 2008).

1. 3. 1. 3. *Coronaviridae*

Coronaviruses (CoV) cause respiratory and/or enteric diseases in a wide variety of species including humans, cattle and pigs (Siddell and Snijder, 2008). Coronaviruses belong to the family *Coronaviridae*, which is subdivided in two subfamilies *Coronavirinae* and *Torovirinae*.

The members of the *Coronaviridae* family are spherical enveloped viruses of 80-220nm in size (Weiss and Navas-Martin, 2005; Maclachlan and Dubovi, 2011). The genome is positive ssRNA (Haagmans and Osterhaus, 2006) and may contain nine to 14 ORFs, (Siddell and Snijder, 2008). It is the largest viral genome among RNA viruses ranging from 26 to 32 kb in length (Lai and Holmes, 2001). It encodes three or four structural proteins: major spike glycoprotein (S), trans-membrane glycoprotein (M and E), nucleocapsid (N), haemagglutinin-esterase (HE) in some viruses and the necessary enzymes for replication.

The different species included in *Coronavirus* genus were organised into three groups, 1, 2 and 3 based on serological and genotypic characteristics (Brian and Baric, 2005; Ziebuhr, 2004). However, novel CoVs have been isolated more recently from both humans and animals (Drosten et al., 2003; Lau et al., 2010). Toroviruses differ structurally and genomically from coronaviruses, (Cavanagh, 2005). The genome of toroviruses consists of a polyadenylated ssRNA molecule of about 25–30 kb (Weiss et al., 1983). The RNA molecule consists of six ORFs, two or which, ORF1a and ORF1b, are large and overlapping, coding for non-structural protein implicated in the replication process. Four open reading frames, ORF2, ORF3, ORF4 and ORF5 code for the spike (S), membrane (M), hemagglutinin-esterase (HE), and nucleocapsid (N) respectively (Snijder and Horzinek, 1993).

Diversity among toroviruses can lead to genetic recombination events, and in fact it was formerly demonstrated that the Bovine torovirus strains identified respectively in Europe (Smits et al., 2003) and Japan (Ito et al., 2007) carried the N gene derived from Pig torovirus, probably by intertypic recombination events due to mixed infection between the two species (Smits et al., 2003).

a. Subfamily *Coronavirinae*

In swine two coronaviruses cause enteritis, Porcine epidemic diarrhoea virus (PEDV) and Transmissible gastroenteritis virus (TGEV) (Chen, 2009; Park et al., 2011a). Clinical signs are similar and it can be difficult to clinically distinguish between them (Wood, 1969; Duarte et al., 1994).

PEDV causes a diarrhoeal disease in piglets known as PED (Porcine epidemic diarrhoea) which was first reported in Belgium and the UK (Wood, 1969; Pensaert and Debouck, 1978). Later, several outbreaks were recorded in numerous pig producing countries (Smíd et al., 1993, Chae et al., 2000; Kim and Chae, 2000; Jinghui and Yijing, 2005; Puranaveja et al., 2009) , and for the first time in America in 2013 (Stevenson et al., 2013).

TGEV causes highly contagious enteritis, characterised by vomiting, watery diarrhoea, dehydration, and death, particularly in pigs under two weeks of age (Saif and Wesley 1999). TGEV was first detected in 1946 in USA (Doyle and Hutchings, 1946) and since then it has been reported in most countries worldwide (Saif and Wesley, 1999). The decreasing prevalence in the last two decades has been ascribed to the emergence of PRCV (Porcine Respiratory Coronavirus), a mutant of TGEV characterised by a large deletion extending from 621 to 681 nucleotides in length localised in the 5' region of the spike (S) protein gene, which leads to a loss of an antigenic determinant (Laude et al., 1993; Vaughn et al., 1995).

A low prevalence has been reported in 1.4% of 639 Korean farms and examined (Chae et al., 2000). More recently, Oh et al. (2011) detected 4.9% of positive samples to TGEV in 1295 pig sera collected to investigate the prevalence of TGEV and PRCV antibodies throughout South Korea.

Bovine Coronavirus (BoCoV) is responsible for enteritis in calves in dairy or beef herds (Clark, 1993), winter dysentery in adult dairy or beef cattle and respiratory disease in adult cattle (Carman and Hazlett, 1992; Storz, 1998). Lin

et al. (2002), referred to coronavirus strains isolated from calves or adult cattle with diarrhoea as bovine enteric or enteropathogenic coronaviruses (BECoV), with strains isolated from cases of pneumonia designated as bovine respiratory coronaviruses (BRCoV). Other authors proposed to subdivide BECoV into BCoV-induced calf diarrhoea (BCoV-CD) and BCoV-winter dysentery (BCoV-WD) (Boileau and Kapil, 2010).

Studies showed that the same virus can cause the simultaneous appearance of enteric and respiratory disease in the same animals (Decaro et al., 2008a, 2008c). However, several studies revealed that gastrointestinal and respiratory coronavirus strains show differences in antigenic, genomic and culture characteristics (Storz et al., 1996; Hasoksuz et al., 1999a, 1999b; Gélinas et al., 2001).

Coronaviruses infecting calves and causing diarrhoea and winter dysentery have been reported in most cattle-producing countries (Bridger et al., 1978, Reynolds et al., 1986; Saif, 1990; Bendali et al., 1999; Jeong et al., 2005; Schroeder et al., 2012).

Levels of reported infection range from 8 to 70% (Langpap et al., 1979; Crouch and Acres, 1984; Uhde et al., 2008). However, it is also important to note that coronaviruses can also be isolated from healthy calves with rates ranging from 0 to 14%, (Bendali et al., 1999; Snodgrass et al., 1986; Gumusova et al., 2007).

b. Subfamily *Torovirinae*

Toroviruses induce mild to profuse diarrhoea in calves (Hoet and Saif, 2004) but also in pigs (Scott et al., 1987) and a variety of other species (Weiss et al., 1983; Beards et al., 1984; Hill and Yang, 1984; Muir et al., 1990)

Bovine Toroviruses (BoTVs) are widespread worldwide (Brown et al., 1987; Durham et al., 1989; Pérez et al., 1998; Haschek et al., 2006), Porcine Toroviruses (PToVs) have not received the same attention as BoTV, although the few existing studies show that the virus is broadly dispersed in pig farms among European, American, African and Asian countries (Penrith and Gerdes, 1992; Pignatelli et al., 2009; Zhou et al., 2013; Anbalagan et al., 2014). The prevalence of both viruses is very variable with recorded prevalence values of 5% (Matiz et al., 2002) to 85% (Alonso-Padilla et al., 2012).

1. 3. 1. 4. *Parvoviridae*

Parvoviruses belong to the family of *Parvoviridae* which is composed of two main subfamilies one of which, *Parvovirinae*, comprises eight genera which infect vertebrates (Cotmore et al., 2014).

In 2014, the taxonomy of the *Parvoviridae* family was reviewed by the members of the ICTV (International Committee on Taxonomy of Viruses) *Parvoviridae* Study Group with proposed changes at the genus and species levels. The genus *Bocaparvovirus* comprises 12 species among which one is Bovine parvovirus and four are Porcine bocaviruses, each with different clades, namely Ungulate bocaparvovirus one (Bovine parvovirus) Ungulate bocaparvovirus 2 (formerly PBoV1, PBoV2, PBoV6), Ungulate bocaparvovirus 3

(previously PBoV5), Ungulate bocaparvovirus 4 (PBoV7) and Ungulate bocaparvovirus 5 (previously PBoV3, PBoV4-1, PBoV4-2). The genus *Copiparvovirus* encompasses Ungulate copiparvovirus 1 (formerly Bovine parvovirus 2) and Ungulate copiparvovirus 2 (previously porcine parvovirus 4 PPV4). Two other genera containing species infecting swine were grouped in *Protoparvovirus* genus (Ungulate protoparvovirus 1, previously known as porcine parvovirus*)* and *Tetraparvovirus* genus (Ungulate tetraparvovirus 1, 2, 3, respectively for Bovine hokovirus 1 and 2 and Porcine hokovirus) (Cotmore et al., 2014).

Parvoviruses are small, non-enveloped, ssDNA viruses, with a genome of approximately 4-6kb that contain terminal palindromic sequences (Tijssen et al., 2011). It comprises two major ORFs, the left ORF encoding non-structural protein NS1 and the right ORF encoding capsid proteins VP1, VP2, and VP3 (Tattersall et al., 1976; Molitor et al., 1983; Ranz et al., 1989; Bergeron et al., 1993; Simpson et al., 2002).

The two ORFs are located in the same frame of the complementary strand as in other autonomous parvoviruses, such as Minute virus of mice (MVM), Canine parvovirus (CPV), Feline panleukopenia virus (FPV), Bovine parvovirus (BPV), and Human parvovirus B19 that encapsidate only the negative sense of the DNA strand. However, other parvoviruses encapsidate different proportions of both negative and positive strands leading to virions with positive and negative DNA strands (Maclachlan and Dubovi, 2011).

Members of *Bocaparvovirus* genus have an additional ORF3 (nuclear phosphoprotein NP1), which is located in the middle of the viral genome between the non-structural and structural-coding regions (Tijssen et al., 2011) 2011).

a. Porcine Parvoviruses

Six phylogenetic groups of parvovirus have been identified in swine (Ni et al., 2014). Reports suggest a worldwide distribution of Parvovirus in swine herds including USA (Xiao et al., 2012, 2013; Zhang et al., 2015), Europe (Szelei et al., 2010; Streck et al., 2013; Gunn et al., 2015) and China (Li et al., 2012; Wang et al., 2014).

The prevalence rates of Porcine parvovirus in domestic pigs populations vary a great deal from 2.09% (Huang et al., 2010) to 58.6% in suckling pigs in China (Li et al., 2012). The latter study stretched over four years and examined a total of 573 clinical samples in five farms situated in three Chinese provinces.

The prevalence of different groups of parvoviruses showed that Ungulate protoparvovirus 1 is moderately prevalent with 25% of pigs detected positive to the virus and also low levels recorded for Ungulate copiparvovirus 2 with 10% (Cadar et al., 2013) both in 120 pig samples from 10 farms covering the western region of Romania. Human parvovirus 4 (Primate tetraparvovirus 1, formerly PARV4) was also isolated form 2.7% of 37 pigs with enteritis (Xiao et al., 2012). Co-infection with two clades of parvoviruses, Ungulate protoparvovirus PPV2 and PPV3, have also been reported with a high infection rate of 79% in domestic pigs in Romania (Cadar et al., 2013).

Porcine bocaviruses (newly named Ungulate bocaparvovirus) were reported by Blomström et al. (2009) in 88% of Swedish pigs from 26 different herds associated with PWMS and 46% without PWMS. In another study, 397 faecal specimens from healthy piglets showed that a lower frequency of 12.6% was positive to Ungulate bocaparvovirus (Cheng et al., 2010). On the African continent and in particular in Uganda, 2.1% of 95 samples collected from six different districts were positive to Porcine bacavirus (Blomström et al., 2013).

Several studies showed that the most prevalent species was Ungulate bocaparvovirus 5 (previously PBoV3) (Ndze et al., 2013; Gunn et al., 2015) but additionally, co-infections with more than one species have been cited with Ungulate bocaparvovirus 1 (formerly PBoV-1) and Ungulate bocaparvovirus 5 being the most prevalent (Choi et al., 2014). Others have uncovered co-infection with four different Porcine bocavirus species (Ndze et al., 2013).

b. Bovine Parvovirus

Bovine Parvovirus (BPV) infection causes diseases of the gastrointestinal and respiratory tracts, foetal infection and reproductive disorders (Durham et al., 1985b; Jordan and Sever, 1994; Manteufel and Truyen, 2009). BPV consists of six species; Ungulate bocaparvovirus 1 (previously bovine parvovirus), Bovine adeno-associated virus, Ungulate erythroparvovirus 1 (bovine parvovirus 3), Ungulate tetraparvovirus 1 and 2 (previously bovine hokovirus 1 and 2) and Ungulate copiparvovirus 1 (formerly bovine parvovirus 2, BPV2) (Cotmore et al., 2014).

Serological investigations and isolation have shown that BPV is widespread in cattle globally (Manteufel and Truyen, 2009) being detected in numerous countries in America, Europe and North Africa (Luo et al., 2013).

Bovine parvovirus (BPV) is highly contagious and spreads rapidly (Luo et al., 2013). Various sero-surveys reported prevalence values ranging from 49 to 86% (Barnes et al., 1982; Sandals et al., 1995). A more recent study using the Loop-Mediated Isothermal Amplification (LAMP) assay for the detection of the virus registered a proportion of 23.1% among 52 diarrhoeic calves originating from several farms from one Chinese province with none of the healthy calves tested being BPV positive (Luo et al., 2013). Earlier, (Durham et al., 1985a) showed that co-infection with intestinal parasites may lead to an enhancement of the mitotic activity in the intestinal epithelium and therefore to a higher BPV infection rate in susceptible cells.

1. 3. 1. 5. *Flaviviridae*

Bovine Viral Diarrhoea virus (BVDV) virus belongs to the family *Flaviviridae*, genus *Pestivirus* that comprises four species, together with Classical swine fever virus and Border disease virus. BVDV includes two species BVDV-1 and BVDV-2 (Pellerin et al., 1994; Ridpath et al., 1994) with 16 subgenotypes in BVDV-1 species (1a to 1p) (Vilcek et al., 2001; Xue et al., 2010) and 2 sub-genotypes in BVDV-2 species (2a and 2b) (Nagai et al., 2004; Xia et al., 2007). BVDV strains have also been classified based on the cytopathic effect (CPE) on cell cultures with BVDV-2 strains more virulent than BVDV-1 viruses (Ridpath, 2010).

The genome is a positive sense, ssRNA which encodes one large polyprotein (van Rijn et al., 1997).

BVDV is prevalent worldwide (Ståhl and Alenius, 2012), and is the cause of many outbreaks in cattle in many countries including Canada (Carman et al., 1998), Spain (Diéguez et al., 2009), USA (Darweesh et al., 2015), Brazil (Lunardi et al., 2008), Poland (Polak et al., 2014), China (Weng et al., 2015) and India (Galav et al., 2007). Virus infection was identified mainly by serology, with prevalence values of 31.6% of individual cows and 80.7% of herds infected in Jordan (Talafha et al., 2009) and 56% of 430 bovine serum samples from 19 farms of one state in Brazil (Canal, Wageck et al., 1998), which appears to be in the prevalence range of European countries (Houe and Meyling, 1991).

In terms of species frequency, BVDV1 strains were more frequently isolated than BVDV2 strains (Ahn et al., 2005). In one study, 77% of isolates belonged to BVDV1 species (Carman et al., 1998). Other authors showed that non-cytopathic isolates accounted for 70% of positive samples to BVDV (Ahn et al., 2005).

BVDV-1 genogroup 1a and 1b were the most common in Europe according to a phylogenetic study carried out in 2001 (Vilček et al., 2001). In the UK, new sub-genotypes have been isolated recently and were demonstrated to belong to type 1d and 1e (Booth et al., 2013), indicating changes within Europe in the last 15 years.

1. 3. 1. 6. *Picornaviridae*

The family of *Picornaviridae* contains 12 genera of small, non-enveloped viruses, with an icosahedral capsid. The genome ranges in length from 7.21-8.45 kb (Racaniello, 2007) is a positive ssRNA, with the 5' end covalently linked to a protein called VPg (Virion protein, genome linked) (Flanegan et al., 1977; Lee et al., 1977) which plays a role in viral life cycle regulation, precisely in priming viral genome replication (Goodfellow, 2011). One ORF encodes the picornavirus polyprotein which is flanked by 5' and 3' UTRs. In swine, picornaviruses are involved in several diseases, including polioencephalomyelitis, reproductive failure, vesicular diseases, myocarditis, pneumonia, diarrhoea and febrile illness (Fenner et al., 1993). Picornaviruses involved in bovine and porcine enteric problems belong to genera *Enterovirus, Kobuvirus, Sapelovirus* and *Teschovirus*.

a. Enterovirus

The *Enterovirus* genus currently contains 12 species, Enterovirus A, B, C and D (Human), Enterovirus E and F (Bovine), Enterovirus G (Porcine), Enterovirus H and J (respectively simian and unclassified simian viruses), Rhinovirus A, B and C (Knowles et al., 2012).

- **Bovine Enterovirus**

During the 1980s, Knowles and Barnett (1985) classified the Bovine enteroviruses (BEVs) into two distinct serotypes, which were later confirmed by the existence of two genetic clusters, designated BEV-A and BEV-B (Zell et al., 2006). BEV-A and BEV-B were found to contain two serotypes and three

serotypes respectively (Zell et al., 2006) which are now named Enterovirus E and Enterovirus F (Knowles et al., 2012).

BEVs can be isolated from healthy cattle or from mild to moderate diarrhoea and reproductive disease (Dunne et al., 1973; 1974; Weldon et al., 1979). They have a global distribution, including USA (Dunne et al., 1974), Australia (Zhang and Burgess, 1986), Germany (Zell et al., 2006), China (Li et al., 2012) and Pakistan (Shaukat et al., 2012). In infected herds rates of infections can be high from 24.6% of 69 fecal samples collected from six major pig-producing Chinese areas (Li et al., 2012) to 50% of calves from an endemically infected herd in USA (Goens et al., 2004) .

- **Porcine Enterovirus**

Porcine enterovirus (PEV), presently comprises six serotypes PEV-9, -10, -14, -15, -16 and OEV-1, a natural interspecies recombinant bovine/porcine enterovirus isolated from sheep (Boros et al., 2011, 2012; Moon et al., 2012). Recently, Ren et al. (2012) performed analyses on the complete genomes of the virus and showed the occurrence of a major recombination event between porcine enterovirus strains leading to the appearence of new species, or possibly intermediate serotypes between PEV-9 and PEV-10. The VP1 protein, situated at the surface of the virion, shows the greatest sequence variability compared to the other parts of the genome (Sozzi et al., 2010), which could explain the recombination event.

Like BEV, Porcine enteroviruses show worldwide dissemination (Knowles et al., 1979; Honda et al., 1990; Ren et al., 2012) with similarly variable prevalence

rates of 25%, where 10 samples were positive among 40 (Sozzi et al., 2010), to another recent study with a rate of 50.2% of 161 samples from 28 farms in the Czech Republic (Prodělalová, 2012).

b. Kobuvirus

Kobuviruses belong to a relatively new genus which was only recognised in 1999 (King et al., 1999) and which contains at least three species, Aichi virus, Bovine kobuvirus, and Porcine kobuvirus (Yamashita et al., 1991; 2003; Reuter et al., 2008).

All kobuviruses have the same genome organisation as other members of *Picornaviridae* except the existence of a leader (L) region following the structural (VP0, VP3 and VP1) and non-structural (2A, 2B, 2C and 3A, 3B, 3C, 3D) regions (Yamashita et al., 2003; ICTV, 2008). The L region was suggested to play a role in viral RNA replication and encapsidation (Sasaki et al., 2003).

- **Bovine Kobuvirus**

Kobuvirus was first isolated from clinically healthy cattle (Yamashita et al., 2003) and five years later from diarrhoeic cattle by Khamrin et al. (2008). As with other Picornaviruses, Bovine kobuvirus is characterised by genetic diversity; a recent finding showed the existence of four phylogenetic lineages of Bovine kobuvirus (Jeoung et al., 2011).

Bovine kobuvirus has been isolated in Europe; Belgium (Mauroy et al., 2009b), Hungary (Reuter and Egyed, 2009) and The Netherlands (Barry et al., 2011), and also Asian countries including Japan (Yamashita et al., 2003), Thailand (Khamrin et al., 2008) and Korea (Park et al., 2011b).

The highest registered prevalence, 78% of nine calf faecal samples, was reported by Barry et al. (2011) and Park et al. (2011b) who described 67% of infected calves; in this study, an overall prevalence of 25.8% of diarrhoeic cattle was reported.

- **Porcine Kobuvirus**

Porcine kobuvirus is a relatively newly discovered species from domestic pigs (Reuter et al., 2008). It has since been isolated in many different parts of the world including Thailand (Khamrin et al., 2009), China (Yu et al., 2009, 2011) Hungary (Reuter et al., 2009, 2010), Brazil, The Netherlands (Barry et al., 2011) and USA (Verma et al., 2013).

The virus isolations can be very frequent in pig farms with the highest isolation rates from young pigs, with figures such as 99% Khamrin et al. (2009) and 84.5% (Park et al., 2010) of pigs with diarrhoea.

c. Sapelovirus

Although Porcine Sapelovirus (PSV) has been associated with asymptomatic infections (Abe et al., 2011), strains have been involved in a broad range of pathologic conditions including diarrhoea, pneumonia and reproductive disorders (Dilovski and Ognianov, 1975; Knowles, 2006; Chen et al., 2012).

PSVs were previously classified in the *Enterovirus* genus as Porcine enterovirus-A (PEV-A) or Porcine enterovirus serotype 8 (PEV-8) (Zell et al., 2001; Krumbholz et al., 2002; Oberste et al., 2003; Tseng and Tsai, 2007).

The genome of PSV is characterised by the L and 2A gene regions which are genetically different from the other porcine enteroviruses (Chard et al., 2006;

Tseng and Tsai, 2007). Sapelovirus genus comprises, Sapelovirus A (formerly Porcine sapelovirus), Sapelovirus B (formerly Simian sapelovirus) and Avian sapelovirus (Tseng and Tsai, 2007). Porcine sapelovirus consists of a single serotype Porcine Sapelovirus-1 (PSV-1) (Cano-Gómez et al., 2013) (formerly known as PEV-8). However, several antigenic variants PEV-8a, PEV-8b and PEV-8c have already been reported (Tseng and Tsai, 2007).

The virus has been isolated in the Czech Republic (Prodělalová, 2012), Spain (Buitrago et al., 2010; Cano-Gómez et al., 2013) and China (Lan et al., 2011) with variable prevalence rates such as 9% of 600 fecal samples collected from a wide territory within Spain (Buitrago et al., 2010), 33.8% (70 among 207 samples) in the Czech Republic (Prodělalová, 2012) and 42.4% among 33 samples in Italy (La Rosa et al., 2006).

d. Teschovirus

Porcine teschovirus (PTV) is the causative agent of a severe encephalomyelitis in pigs of all ages causing considerable economic losses (Salles et al., 2011) (Harding et al., 1957). They are ubiquitous in the environment (Mahnel et al., 1977), and have also been isolated from faeces of healthy pigs (Forman et al., 1982) but also from cases of diarrhoea (Wang et al., 2010; Zhang et al., 2010).

The genus *Teschovirus* consists of 13 serotypes of one single species, PTV (Zell et al., 2001; Cano-Gómez et al., 2011; Boros et al., 2012b). Several authors have previously reported recombination occurring within the genus (Heath et al., 2006; Simmonds, 2006). Different studies showed a variation in prevalence of the 1 to 10 serotypes identified, with Porcine teschovirus serotype 8 (PTV-8)

being the most common (Lin et al., 2010; Sozzi et al., 2010; Zhang et al., 2010; Cano-Gómez et al., 2011a).

Porcine teschoviruses are distributed world-wide in pig producing countries including China (Zhang et al., 2010; Wang et al., 2013), Italy (La Rosa et al., 2006), USA (Pogranichniy et al., 2003), Africa and Australia (Forman et al., 1982) with variable rates of isolation ranging from 11% of 525 fecal samples collected from 21 counties and cities in Taiwan (Lin et al., 2010). Also in this country the serological prevalence was 70% of pigs. Wang et al. (2013), indicated a rate of 47% of positives (among a total of 127 samples) detected by PCR, although the same samples were 90% serologically positive to teschovirus. It is also important to point out that high rates of isolation from healthy pigs have also been reported (Sozzi et al., 2010; Prodělalová, 2012).

1. 3. 1. 7. *Reoviridae*

The *Reoviridae* family infect a wide range of animal species producing a number of pathologies including enteritis. There are two subfamilies: *Sedoreovirinae* and *Spinareovirinae* with respectively six (*Orbivirus, Rotavirus, Seadornavirus, Phytoreovirus, Cardoreovirus and Mimoreovirus*) and nine (*Orthoreovirus, Aquareovirus, Oryzavirus, Fijivirus, Mycoreovirus, Cypovirus, Idnoreovirus, Dinovernavirus and Coltivirus*) genera (MacLachlan and Dubovi, 2011). Members of genera *Rotavirus* and *Orthoreovirus* are involved in intestinal infections.

The difference between *Sedoreovirinae* and *Spinareovirinae* subfamilies reside in the absence of spike protein in each of the icosahedral vertices in the former and its presence in the later (Quinn et al., 2011).

Members of this family are non-enveloped dsRNA viruses (Dai et al., 2012). They may contain 9 to 12 genome segments with a genome size of 19 to 32 kb (Mertens, 2004; Attoui et al., 2005).

The orthoreovirus genome is organised in 10 segments, three large (L1, L2 and L3), three medium (M1, M2 and M3), and four small (S1, S2, S3, and S4) segments (Shatkin et al., 1968; Watanabe et al., 1968; Nibert and Schiff, 2001; Schiff et al., 2007). Each segment encodes a single protein with the exception of S1 which encodes two proteins (Jacobs and Samuel, 1985; Sarkar et al., 1985). A total of 12 proteins are encoded by the 10 RNA segments including eight structural proteins (λ1, λ2, λ3, σ1, σ2, σ3, μ1 and μ2) and four non-structural proteins (μNS, μNSC, σNS and σ1S) (Schiff et al., 2007).

Rotaviruses comprise 12 segments that code for 6 structural proteins (VP1 to VP4, VP6 and VP7) and five or six non- structural proteins (NSP1 to NSP6) (Estes and Kapikian, 2007). Like orthoreoviruses, each segment encodes a unique protein, except segment 4 (VP4) that cleaves into two fragments (VP5 and VP8) (Gilbert and Greenberg, 1998) and segment 11, which encodes NSP5 and in some strains also NSP6 (Campagna et al., 2005).

a. Rotavirus

Rotaviruses cause acute watery dehydrating diarrhoea in several host species (Parashar et al., 2006). The *Rotavirus* genus encompasses eight serogroups (A

to H) based on the serological reactivity of the intermediate layer of the virus capsid VP6 (Hoshino and Kapikian, 2000). Rotavirus A, B, C and H infect humans and animals whereas rotavirus D, E, F, and G infect only animals (Matthijnssens et al., 2012; Molinari et al., 2014).

The binary system of classification of rotaviruses is based on the neutralisation specificity of the outer capsid VP4 referred to as P, because it is protease-sensitive, and VP7, stated as G because it is a glycoprotein (Coulson, 1996). More recently, group A rotaviruses have been classified based upon the sequence of VP4 and VP7 genes and 27 G genotypes (G1 to 27) and 35 P genotypes (P[1] to P[35]) have been described (Matthijnssens et al., 2011). Genotypes G2, G3, G4, G5, G9, G11 and P[6], P[7], P[13], P[19], P[23], P[26] and P[27] have been described worldwide in porcine species (Martella et al., 2010; Malik et al., 2014). In bovine species, genotypes G6, G8, G10 and P[1], P[5], P[11], P[15] and P[21] (Martella et al., 2010) were epidemiologically prevalent, with the single predominance of genotype G5 P[7] in porcine and G6 P[5] in bovine populations (Papp et al., 2013).

It is important to note that several G and P types are common between rotaviruses infecting host species indicated by molecular analysis which showed shared sequences (Martella et al., 2010). Indeed, the segmented nature of the rotavirus genome has allowed assortment events during multiple infection with different strains (Maunula and Von Bonsdorff, 2002). For example, it has been reported that porcine-like rotavirus A G5 P[7] strain was detected in cattle and reciprocally bovine-like rotavirus A G6P[1] strain was

isolated from pigs (Lorenzetti et al., 2011). In another study that characterised the P and G genotypes of bovine rotavirus A, 30 G8 and G6 Rotavirus A strains contained 1-9 segments of genome that have porcine or human origin (Park et al., 2011). Also, in one study, there was evidence of segment exchange between rotaviruses group A and group D (Trojnar et al., 2010), which can eventually lead to zoonotic transmission (Luchs and Timenetsky, 2014) or the appearance of novel strains (Papp et al., 2013).

Papp et al. (2013) analysed 55 published articles about rotavirus A in cattle and pigs and concluded that genotypes G and P were more diverse in pigs than in cattle and comparable to the diversity observed in human genotypes (Gentsch et al., 2005; Bányai et al., 2012).

Group A rotaviruses are very prevalent in mammals and birds (Estes and Kapikian, 2007) and have been associated with enteritis in calves (Saif and Jiang, 1994; Saif et al., 1994) and post-weaning enteritis in piglets (Saif and Jiang, 1994; Kapikian and Chanock, 1996). Rotaviruses B and C are equally important in the aetiology of enteritis in pigs and cattle and have both been reported in severe diarrhoea (Saif and Jiang, 1994; Tsunemitsu et al., 1992; 2005; Smitalova et al., 2009), although group C rotavirus are more frequently isolated (Geyer et al., 1996; Smitalova et al., 2009).

b. Orthoreovirus

Orthoreoviruses comprise five species (I to V), species I comprising four mammalian serotypes (1 to 4) (Duncan et al., 2004; Fauquet et al., 2005). Species I (Mammalian orthoreovirus) is divided into four serotypes based on

neutralisation and haemagglutination inhibition assays (Sabin,1959; Rosen, 1962; Stanley, 1967). Porcine orthoreoviruses cause enteritis in piglets (Dai et al., 2012) and experimentally serotype I infection has been shown to cause swine enteritis and pneumonia (Kasza, 1970; Baskerville et al., 1971).

Mammalian orthoreoviruses are isolated worldwide with porcine orthoreovirus type 3 isolated from diarrhoeic pigs in China, South Korea (Zhang et al., 2011; Kwon et al., 2012) and USA (Narayanappa et al., 2015).

Orthoreoviruses are isolated with variable frequency in outbreaks ranging from 19% of 237 fecal samples collected from 78 Korean pig farms. (Kwon et al., 2012) to 37.5% of 48 fecal samples originating from three American states which were positive to a novel orthoreovirus (Thimmasandra Narayanappa et al., 2015).

1. 3. 2. Bacteria

Among bacterial pathogens implicated in cattle and pig intestinal infections, several key species are members of the Enterobacteriaceae family including *Escherichia, Salmonella* and *Yersinia*. Other pathogenic genera include *Clostridium, Campylobacter, Lawsonia, Bacteroides* and *Brachyspira*.

Among bacterial virulence determinants, toxins play a major role in the pathogenicity level of a strain. Toxins can target the cellular membrane and damage its structure, they include phospholipases, lysins and hemolysins, or they can interact intracellularly with a cellular target. Most of enterotoxins targeting the alimentary tract are proteins formed by two (binary toxins) or three (tripartite toxins) protein chains that include a binding and an enzymatic

subunit (Popoff, 2011). Others, such endotoxins generally are secreted by Gram negative bacteria and consist of a lipopolysaccharide (LPS) that is released after lysis of the bacteria (Lubran, 1988), in which lipid A is the toxic component.

After binding to the enterocyte, the mechanism of action of toxigenic *E. coli* enterotoxin consists of an increase of cAMP which results in an increase of the secretion of water and electrolytes and decrease of the assimilation of sodium chloride. Disruption of the intestinal homeostasis can also be the result of toxins that alter the intracellular junction of the intestinal cell, leading to an increased permeability and an important loss of fluids. Malabsorption is also the result of the destruction of the intestinal villi, an important component of nutrient absorption.

1. 3. 2. 1. *Escherichia coli*

Pathogenic *Escherichia coli* are divided into seven pathotypes, namely Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC) that belongs to the Verotoxin *E. coli* (VTEC) also referred to as Shigatoxin *E. coli* (STEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Necrotoxic *E. coli* (NTEC) and Diffusely adherent *E. coli* (DAEC). Recently a new pathotype has been described following genome sequence analysis of *E. coli* isolated from an outbreak in Germany, the authors suggesting the appellation Entero-Aggregative-Haemorrhagic *E. coli* (EAHEC) (Brzuszkiewicz et al., 2011). In calves and piglets, the most frequent cause of colibacillosis is ETEC (Younis et al., 2009); this was

also confirmed by a meta-analysis study of 60 years of research about pathogenic *E. coli* in calves (Kolenda et al., 2015). In diarrhoeic calves ETEC with fimbrial type F5 and F41 are most commonly detected (Kolenda et al., 2015). Less frequent causes of diarrhoea are EHEC including O157:H7 (Daniel et al., 1998; Kang et al., 2004) and EPEC (Foster and Smith, 2009) although EHEC has also been demonstrated to be carried by healthy cattle (Moxley, 2004). STEC isolates have also been implicated in neonatal diarrhoea (Sandhu and Gyles, 2002).

In pigs, ETEC expressing fimbrial types F4, F5, F6, F14, F18 and F41 is a common enteric pathogen in young piglets (Nagy and Fekete, 1999; Vu-Khac et al., 2007). After weaning, pigs are commonly infected with *E. coli* fimbrial type F18 (Jensen et al., 2003).

E. coli pathotypes EPEC and EHEC/STEC belong to the group of attaching-effacing (AE) *E. coli*, producing an unique attachment pathology with effacement of the microvilli of the intestinal cell (Wales et al., 2005).

ETEC produce four enterotoxins, heat-labile enterotoxin (LT), heat-stable enterotoxin (STa and STb) (Levine, 1987) and enteroaggregative heat-stable toxin 1 (EAST1) (Savarino et al., 1996; Nataro and Kaper, 1998), which induce increased fluid secretion and reduced absorption. STEC produce at least one of the shiga toxins Stx1 or Stx2 (Jothikumar and Griffiths, 2002). However, when they cause AE lesions, they are classified as the EHEC pathotype.

Virulence genes harboured by *E. coli* made their classification complex with many studies describing controversial results as a result of interactions between pathotypes leading to overlapping virulence markers.

1. 3. 2. 2. *Salmonella*

Of the six sub-species of *Salmonella enterica, S. enterica* subsp. *enterica* and to a lesser extent *S*. *enterica* subsp. *arizonae* and *S. enterica* subsp. *diarizonae*, are responsible for most infections of livestock (Fookes et al., 2011). The vast majority of serovars belongs to *S. enterica* subsp. *enterica*.

Pathologically, *Salmonella* produce typhoid fevers or non-typhoid gastroenteritis (Langridge et al., 2008). Typhoid serovars produce systemic diseases in a narrow range of host species and enteritis is generally a feature at certain stages of this type of infection (Barrow and Duchet-Suchaux, 1997). Non-typhoid *Salmonella* serotypes affect a wide range of host species colonising the gastrointestinal tract, and generally with limited systemic involvement (Tauxe and Pavia, 1998).

The virulence determinants of *Salmonella* which differentiate it from other closely related members of the Enterobacteriaceae represent a wide matrix of genetic elements including chromosomal *Salmonella* Pathogenicity Islands (SPIs) (Eswarappa et al., 2008), virulence plasmids (Rotger and Casadesús, 1999) and phages (Moreno Switt et al., 2013). They also include integrons which are mobile genetic elements into which gene cassettes are inserted and which have been associated with multidrug resistance (Ribeiro et al., 2011).

Salmonella enterica serovars associated with systemic disease in livestock such as pigs and cows, include Enteritidis, Typhimurium, Choleraesuis and Dublin possess virulence plasmids (VPs) (Nakamura et al., 1985; Gulig and Curtiss, 1987; Danbara et al., 1992; Libby et al., 1997) the essential element of which is a highly conserved 8 kb region (Guiney and Fierer, 2011) containing the *spvRABCD* (*Salmonella* Plasmid Virulence) and *mig-5* (macrophage-inducible gene coding for a putative carbonic anhydrase) are present in all plasmids.

Twenty three *Salmonella* Pathogenicity Islands (SPI) have been described (Hayward et al., 2013) and are differentially distributed among *Salmonella* serovars. Thus *S.* Typhimurium expresses SPI-1-6, 9, 12-14 and 16, whereas *S.* Enteritidis expresses SPI-1-6, 9, 10, 12-14, 16, 17 and 19. Serotypes Derby and Dublin contain SPI-1-5. SPI-7 which secretes the Vi antigen, typical of *S*. Typhi, was not detected (Litrup et al., 2010). All strains virulent for animals thus contain SPI-1 and SPI-2.

SPI-1 and SPI-2 play a role in the penetration of intestinal cells and multiplication in macrophages respectively (Juhas et al., 2009). Not all virulence effectors have been characterised or are understood, Heffron et al. (2011) indicated that >40 virulence factors have been identified. SPI-1 together with products of SPI-5 are responsible for the pathogenesis of enteritis.

Adhesion to host cells is mediated by fimbriae and non-fimbrial adhesins (Guo et al., 2007). *S.* Typhimurium, Enteritidis and other serovars possess up to 13 independent fimbriae (Thomson et al., 2008), although the function of each has not yet been clarified. In *Salmonella* non-fimbrial adhesin (SiiE) has been

recently described (Wagner et al., 2011; Griessl et al., 2013), which is secreted by the Type 1 secretion system (T1SS) and helps the installation of the Type three secretion system (T3SS) on the host cell apical membrane (Barlag and Hensel, 2015).

1. 3. 2. 3. *Yersinia enterocolitica*

Yersinia enterocolitica contains six biotypes based on biochemical tests (1A, 1B, 2, 3 4 and 5) and divided in 60 serotypes based on the cell wall LPS (lipopolysaccharide) (Skurnik et al., 1999).

Many strains of *Y. enterocolitica* can infect pigs, with serovar 3 isolated more frequently than other serotypes (Toma and Deidrick, 1975; Fukushima et al., 1984). *Y. enterocolitica* serotype O:3 was found in 3.3% of pigs and 0.5% of cattle (Simonová et al., 2007).

Chromosomal genes *ail, rfbC*, and *yst* determine pathogenicity in *Yersinia*, determining adhesion to host cells and resistance to the bactericidal effect of complement (Platt-Samoraj et al., 2006). *Yersinia* plasmid pYV (Plasmid for *Yersinia* Virulence), contains virulence genes yadA (*Yersinia* adhesion A) and virF, respectively, coding for the YadA membrane protein that protects the bacteria against leucocyte activity (Ruckdeschel et al., 1996) and for the transcription of Yop protein which protects bacterial cells from macrophage activity (Michiels et al., 1990). However, for screening purposes, Thoerner et al. (2003) suggested the use of plasmid virulence genes with at least one chromosomal virulence gene.

Other virulence factors have been reported including a heat stable enterotoxin YstA (Pai and Mors, 1978; Platt-Samoraj et al., 2006) and more recently a type VI secretion system (Jaakkola et al., 2015).

1. 3. 2. 4. *Clostridium*

Clostridia are Gram positive, anaerobic, spore-forming bacilli. Two species are of clinical relevance for pigs and cattle, *Clostridium difficile and Clostridium perfringens*.

a. *Clostridium difficile*

C. difficile is a taxonomically heterogeneous group which phylogenetically is closer to the members of *Peptostreptococcaceae* (Collins et al., 1994; Ludwig et al., 2009) and will in the future be called *Peptidoclostridium* (Yutin and Galperin, 2013).

Toxigenic strains of *C. difficile* have been reported to cause severe enteric diseases in calves (Rodriguez-Palacios et al., 2006) and pigs (Songer et al., 2000). Two exotoxins have been identified in *C. difficile*, toxin A, an enterotoxin, and toxin B, a cytotoxin, respectively coded by *tcdA* and *tcdB* genes (Kuehne et al., 2010). Both toxins cause colonic inflammation and epithelial tissue damage leading to diarrhoea (Voth and Ballard, 2005). Some *C. difficile* strains also produce a binary toxin (Perelle et al., 1997), fimbriae (Goulding et al., 2009) and the fibronectin binding protein FbpA (Barketi-Klai et al., 2011) involved in cellular colonisation.

The prevalence of *C. difficile* was investigated in 144 calves with diarrhoea and 134 without diarrhoea from 102 Canadian dairy farms, with 7.6% of diarrhoeic

and 14.9% of non-diarrhpeic calves positive to *C. difficile,* although the presence of toxins was significantly higher in diarrhoeic than non-diarrhoeic samples (Rodriguez-Palacios et al., 2006). In another study more than 25% of 253 calves presenting a diarrhea from six different states in America were infected with toxigenic *C. difficile*. In this study, PCR was used to screen the samples, unlike the previous study where ELISA was performed for the detection (Hammitt et al., 2008). In pig enteritis, the prevalence of *C. difficile* is generally higher as it is one of the most important cause of neonatal diarrhea in piglets. It has been reported a prevalence of 90% in piglets (185 neonatal piglets from 3 farms) with moderate to severe scouring (Squire et al., 2013). A variable prevalence in both pigs and cattle without diarrhoea has also been reported (Hammitt et al., 2008; Norman et al., 2009)

b. *Clostridium perfringens*

C. perfringens is a major enteropathogen in many livestock species (Lebrun et al., 2007). *C. perfringens* can produce 17 toxins (Li et al., 2013), not all of which are produced by all strains. *C. perfringens* is classified into five toxinotypes A-E based on the production of four major toxins, α, β, ε and ι (Hatheway, 1990). Some strains secrete additional toxins such as enterotoxin (Cpe) (Sarker et al., 1999), beta2 toxin (cpb2), necrotic enteritis B-like toxin NetB and TpeL (Li et al., 2013).

The Cpe toxin has been identified in strain of type A, C, D and E, but not in type B (McDonel, 1980; Sayeed et al., 2010). NetB toxin has been identified in avian

C. perfringens A strains (Keyburn et al., 2008). The TpeL is thought to enhance virulence of necrotic enteritis of avian strains (Coursodon et al., 2012).

Strains of *C. perfringens* types A and C are the most commonly isolated from pigs (Songer and Uzal, 2005). A higher prevalence of *C. perfringens* type A was reported in suckling pigs by Chan et al. (2012), which, interestingly, harboured NetB and TpeL genes and other studies described 30 - 100% morbidity and mortality in pigs infected with *C. perfringens* C (Songer and Glock, 1998; Songer and Uzal, 2005).

Similarly, C. *perfringens* A and C have been implicated in enteritis in cattle (Songer, 1996) with Bartels et al. (2010) reporting 54% of 424 calves (from 108 dairy herds) infected by *C. perfringens* and in another study, Pardon et al. (2012) described evidence of enterotoxaemia in 10% of 3519 veal calves from 10 cohorts, which was mainly due to *C. perfringens* type C.

1. 3. 2. 5. *Campylobacter*

The *Campylobacter* genus comprises Gram negative, spirally curved rod, non-spore forming bacteria. Several species of *Campylobacter* can cause gastroenteritis in human and animals, but particularly *C. jejuni* and *C. coli* (Nietfeld, 2013).

Several virulence genes playing a role in the pathogenicity of *Campylobacter* spp. have been identified, including the *flaA* gene involved in motility, *cadF*, demonstrated to determine adhesion, *cdtB*, responsible for the production of cytolethal distending toxin and *iam* for invasiveness (Krutkiewicz and Klimuszko, 2010; Rizal et al., 2010). Previously, other toxins have been

described in *Campylobacter* spp. species, notably enterotoxin, haemolytic toxins, shiga-like toxin and hepatotoxin (Wassenaar, 1997).

a. *Campylobacter jejuni*

C. jejuni comprises two subspecies, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* (Parker et al., 2007). *C. jejuni* is isolated more commonly than *C. coli* from calves (Selwet and Galbas, 2012) causing diarrhoea (Schulze, 1992), although in one study, 15% of calves were infected with *C. jejuni* with no difference in detection rates between calves with or without diarrhoea. However, diarrhoeic calves were shedding the bacteria more often than healthy calves (Klein et al., 2013). In pigs it has been isolated with a rate of 25% of individuals with diarrhoea (Burrough et al., 2013).

b. *Campylobacter coli*

C. coli has long been recognised as a major porcine enteropathogen (Andress et al., 1968; Vandamme and On, 2001). Although, *C. coli* has been isolated from pigs with severe diarrhoea several authors question the role of *C. coli*, with experimentally infected pigs remaining healthy (Bratz et al., 2013). *C. coli* has been recovered from 75% pigs with recognised colitis lesion (Burrough et al., 2013), although it has also been identified in healthy pigs (Oporto et al., 2007; Denis et al., 2011). A low incidence 9.5% has been reported in calves (Adesiyun et al., 1992).

1. 3. 2. 6. *Bacteroides fragilis*

Bacteroides fragilis is a non-spore forming, Gram negative, obligately anaerobic, curved bacillus, normally regarded as a normal component of the gut flora of the intestine of human and animals (Collins et al., 1989).

Enterotoxigenic *Bacteroides fragilis* (ETBF) can cause diarrhoea in animals, children and adults (Sears, 2009). A toxin (known as fragilysin) is encoded by the *bft* gene located in a pathogenicity island (BfPAI) (Franco et al., 1999), although many *B. fragilis* strains are non-toxigenic (NTBF) and are found in healthy individuals.

Oral administration of ETBF to gnotobiotic pigs resulted in watery diarrhoea with intestinal lesions (Duimstra et al., 1991) with similar symptoms also reported in naturally infected pigs (Collins et al., 1989). In experimentally infected calves ETBF, has produced fluid accumulation in ligated intestinal loops (Myers et al., 1985).

1. 3. 2. 7. *Brachyspira*

Members of *Brachyspira* genus are Gram negative and anaerobic (Euzéby, 2013) containing two porcine enteropathogens, *B. hyodysenteriae* and *B. pilosicoli* (Hudson et al., 1976; Thomson et al., 1997). Phenotypically the two species can be differentiated by the strength of beta-haemolysin that is strong in *B. hyodysenteriae* and fairly weak in *B. pilosicoli* (Fellström and Gunnarsson, 1995). A recent study has shown a strong correlation between the degree of beta-haemolysis and the potential for virulence in *Brachyspira* spp. (Burrough et al., 2012).

a. *Brachyspira hyodysenteriae*

B. hyodysenteriae is one of the agents responsible for swine dysentery (Taylor and Alexander, 1971), which is a mucohaemorrhagic enteric disease.

Many genes have been implicated in its virulence including *tlyA–C* and *hlyA* coding for beta-haemolysin and strong haemolysin respectively (Barth et al., 2012), *nox* encoding NADH oxidase (Stanton et al., 1999), the *vsp* genes encoding surface mediated proteins (Witchell et al., 2011) and a set of genes coding for a lipo-oligosaccharide (Halter and Joens, 1988). More recently, La et al. (2011) have demonstrated that *B. hyodysenteriae* has a 36 kb plasmid comprising several virulence genes that may also contribute to its virulence (Jensen and Stanton, 1993) with strains lacking the plasmid being avirulent (Achacha et al., 1996). The plasmid encloses genes that encode rhamnose biosynthesis enzymes and other glycosyltransferases (Nuessen et al., 1983).

Detection frequency of *B. hyodysenteriae* was reported to vary between studies, Novotná and Škardová (2002) indicated 44% in 100 diseased pigs, whereas Baccaro et al. (2003), registered a rate of 1.4% in 541 pigs from 70 swine herds. In another study involving 462 faecal samples from 43 herds showed a prevalence of 37.2% (Suh and Song, 2005).

b. *Brachyspira pilosicoli*

B. pilosicoli produces intestinal spirochaetosis in pigs (Trott et al., 1996), and has been recognised as an important cause of colitis and typhlitis (Hampson et al., 2006) a less severe disease compared to swine dysentery (Trott et al., 1996).

The prevalence of infection has been reported to vary between 1% to 20% in different studies and countries (Baccaro et al., 2003; Oxberry and Hampson, 2003; Calderaro et al., 2006).

1. 3. 2. 8. *Lawsonia intracellularis*

L. intracellularis is a Gram negative, obligately intracellular bacteria involved in proliferative enteritis, mainly in grower-finisher pigs (McOrist and Gebhart, 2006) which is characterised by morphological changes of the intestinal mucosa due to enterocyte hyperplasia (Nietfeld, 2013). Affected pigs present with signs of bloody diarrhoea and sometimes sudden death (McOrist and Gebhart, 1999).

The prevalence of *L. intracellularis* in pigs with diarrhoea has been estimated to vary generally between 3.3% and 20% (Kim et al., 1998; Baccaro et al., 2003; Suh and Song, 2005). Although higher rates of around 70% have also been reported but were mainly seroprevalence studies (Marsteller et al., 2003; Stege et al., 2004).

1. 3. 3. Parasites

1. 3. 3. 1. *Trichuris suis*

Trichuris suis, more currently known as whipworm, is a helminth parasite (Horton, 2014) infection which is usually asymptomatic, but in large numbers, they can cause watery diarrhoea containing blood, anorexia with retarded growth (Caron et al., 2014).

Morphologically, infected pigs showed a haemorrhagic colon surface with increased mucus production (Li et al., 2012) with sometimes a disruption in the intestinal mucosal layer accompanied by profound histopathological changes (Mansfield and Urban, 1996). A synergistic effect has been observed between *T. suis* and other pathogens such as spirochaetes (Rutter and Beer, 1975) or *Campylobacter* (Mansfield et al., 2003).

Prevalence rates have been reported to vary according to countries from 11.1% to 25% (Eijck and Borgsteede, 2005; Matsubayashi et al., 2009; Nissen et al., 2011). Lower percentages of 0.5% (of a total of 200 pigs), 2.9% (of 384 pigs) and 5% (of 920 pigs) have also been observed in certain African countries (Atawalna et al., 2015; Jufare et al., 2015; Olaniyi et al., 2016).

1. 3. 3. 2. *Cryptosporidium*

Cryptosporidium is a protozoan that belongs to the *Cryptosporidiidae* with 23 recognised species in the genus (Xiao, 2010). Cryptosporidiosis is also zoonotic (Tzipori, 1983). Studies have shown that *C. suis* and pig genotype II are prevalent in pigs. Genotype II has been identified in pigs following 18S rRNA sequencing and phylogenetic analysis that showed two different genotypes (Ryan et al., 2004), *C. suis* being genotype I. Genotype II *Cryptosporidium* have been found exclusively in adult pigs and proposed to be named *C. scrofarum* in 2013 (Kváč et al., 2013). *C. parvum* has been decribed to be prevalent in cattle (Morgan et al., 1998; Ryan et al., 2004). Cattle can also be infected with *C. andersoni* and *C. bovis* (Lindsay et al., 2000; Santín et al., 2004). Cattle and porcine *Cryptosporidium* species are not necessarily host-specific (Fayer, 2010).

In calves and piglets, *Cryptosporidium* causes diarrhoea and severe intestinal lesions (Angus, 1983). In calves, many recent studies suggested a prevalence rate of 16% (461 calves from different farms) (Maurya et al., 2013), 22% (364 faecal specimens from five farms) (Ng et al., 2011) and 39% (456 faecal samples from different farms) (Venu et al., 2013). In pigs and piglets almost similar rates have been reported (Nguyen et al., 2012; Maurya et al., 2013; Petersen et al., 2015).

1. 3. 3. 3. *Giardia*

The genus *Giardia* is another protozoan that infects numerous hosts. *G. duodenalis* has been associated with beef and dairy calf diarrhoea (O'Handley et al., 1999; Olson et al., 2004). Based on the genetic and morphological features of the parasite, *G. duodenalis* is a complex species formed of 8 different taxonomic groups called assemblages (A to H) (Monis et al., 2009; Lasek-Nesselquist et al., 2010). Assemblage E seems to be the most prevalent in calves (Thompson, 2004). Few studies have reported on the presence of *Giardia* in pigs compared to cattle, however, assemblages A and E have been cited with E being the most common (Feng and Xiao, 2011).

In naturally infected calves, *Giardia* causes villous atrophy of the intestine leading to diarrhoea with mucus (Ruest et al., 1997). Herd prevalence were usually relatively high, 18-84% for pigs and 60-100% for cattle (Maddox-Hyttel et al., 2006). Animal-level prevalence ranged from 9% to 14% in pigs (Olson et al., 1997; Petersen et al., 2015) and 7% to 27% in cattle (Ng et al., 2011; Gillhuber et al., 2014).

1. 3. 3. 4. *Eimeria*

Eimeria species are coccidian parasites that infect the intestinal tract in cattle (Lucas et al., 2014). Symptoms of coccidiosis in cattle consist of watery diarrhoea (Daugschies and Najdrowski, 2005), weight loss, apathy, reduced weight gain and dehydration (Stockdale et al., 1981; Jolley and Bardsley, 2006). Among the 20 described *Eimeria* species in cattle, two are pathogenic; *E. bovis* and *E. zuernii* (Seppä-Lassila et al., 2015), and are frequently associated with cattle eimeriosis (Enemark et al., 2013). Prevalence of *Eimeria* spp. in calves is usually high, 13% to 84% as reported (Koutny et al., 2012; Enemark et al., 2013; Gillhuber et al., 2014; Seppä-Lassila et al., 2015). *Eimeria* are relatively infrequently isolated from porcine enteritis (Karamon et al., 2007), with recent reported prevalence values of 8% and 13% (Zhang et al., 2012; Ruiz et al., 2016).

1. 3. 3. 5. *Isospora suis*

Isospora suis is another coccidian enteropathogen in pigs known to produce diarrhoea in suckling piglets (Niestrath et al., 2002; Karamon et al., 2007). Isosporosis manifests with diarrhoea, dehydration and growth retardation (Gualdi et al., 2003) mainly in pigs of less than three weeks old (Worliczek et al., 2009). *I. suis* was reported to be highly prevalent in intensive pig production (Mundt et al., 2005). It was detected with a relatively high prevalence in many studies with a reported rate of 17% (779 litters of suckling piglets collected from 80 farms in 17 provinces in China) (Zhang et al., 2012). In a cohort study covering 12 European countries, the prevalence of the parasite in the litters was 26% and the herd prevalence was 69% (Torres et al., 2004).

1. 4. Mixed enteric infection in cattle

Investigations on the detection of pathogens in infectious enteritis have most frequently concerned specific individual pathogens. However, the involvement of several organisms in disease initiation has been reported and should be considered in every case of enteritis. Pathogen combinations may include several species of viruses, bacteria or parasites. Mixed infections are typically associated with more severe disease (Reynolds et al., 1986). Hoet and his colleagues reported that calves infected with two or more pathogens were six times more likely to develop clinical diarrhoea compared with calves that shed only one pathogen (Hoet et al., 2003).

There are a number of reports of mixed infections. Thus, ETEC and *Salmonella* spp. have been isolated simultaneously from diarrhoeic calves (Bendali et al., 1999; Hoet et al., 2003). In Rotavirus A infections, co-infections may aggravate the outcome of the disease (García et al., 2000). Rotavirus, Coronavirus and *Cryptosporidium* (de la Fuente et al., 1998) or Rotavirus, Coronavirus, *Cryptosporidium* and *E. coli* (Uhde et al., 2008; Torsein et al., 2011) can simultaneously co-infect the intestine leading to an exacerbated clinical picture. Swiatek et al. (2010) showed that in rotavirus infections, diseased calves could be infected simultaneously with more than one strain of rotavirus. Calves persistently infected with BVDV may have a higher death loss at a young age when challenged with other enteric pathogens (Blanchard, 2012). Bovine norovirus-infected calves have tested positive to other viruses and bacteria including bovine enteric Nebraska-like calicivirus, Bovine rotavirus A, B and C,

Bovine coronavirus, Bovine torovirus, BVDV and shiga-toxin-producing *E. coli* (Park et al., 2007).

In the case of distantly unrelated pathogens the most frequently observed co-infection in calves involves *Cryptosporidium* and Rotavirus, followed by concomitant infection with *Cryptosporidium*, Rotavirus, *E. coli* and/or *Salmonella* (García et al., 2000).

1. 5. Mixed enteric infection in pigs

Reports of co-infected pigs with two species of viruses have been described. PEDV-induced diarrhoea in neonatal piglets has occurred with Porcine group A rotavirus with the co-infection being more severe and prolonged when compared to single infections (Jung et al., 2008). Porcine bocaviruses have also been isolated simultaneously with PCV2 in diseased pigs (Blomström et al., 2009, 2010; Zhai et al., 2010; Cadar et al., 2011; Shan et al., 2011; Zeng et al., 2011; Zhang et al., 2011).

Sapovirus infections together with other enteric pathogens, such as Group A and C Rotaviruses, may play a role in the aetiology of weaning and post-weaning enteritis of piglets (Gouvea et al., 1991; Saif et al., 1994; Will et al., 1994; Martella et al., 2008).

Mixed porcine infection with bacteria and viruses are well illustrated by *L. intracellularis* which increases the severity of PCV2 infections (Allan et al., 2004), with a specific synergy suggested between these two pathogens (Jensen et al., 2006). PCV2 has also been found in pigs co-infected with *S.* Choleraesuis (Murakami et al., 2006; Kawashima et al., 2007). Combinations of different

bacteria have also reported, *L. intracellularis* was isolated in a co-infection with *E. coli* (Schauer et al., 1998; Boesen et al., 2004), a dependency suggested by the inability to establish experimental infection of gnotobiotic pigs with *L. intracellularis* alone (McOrist et al., 1993). Moreover, a recent study showed that when pigs were challenged with *L. intracellularis* concomitantly with *S.* Typhimurium, the rate of *Salmonella* shedding was higher compared to the control group (Borewicz et al., 2015), which confirms the suggestion that *L. intracellularis* might predispose to a longer carriage of *S. enterica* (Beloeil et al., 2004).

Infection involving two species from the same genus has also been described; in this case co-infection with two *Salmonella* serovars, *S*. Typhimurium and *S*. Choleraesuis in swine herds (Ha et al., 2005).

1. 6. Relationship between the presence of pathogens and the onset of enteritis

A primary process in a case of enteritis is the appearence of diarrhoea sometimes with other symptoms such as fever, asthenia, or watery and bloody faeces which can provide an indication of the extent of the invasiveness of the causal agent. The association of the symptoms and the general condition of the animal with the isolation of an enteric pathogen from a faecal specimen is indicative evidence of the cause-effect principle. The screening of a faecal sample for the identification of pathogens by PCR indicates solely the presence or the absence of a particular microorganism. However, other methods such as faecal culture recovers enteric pathogens that can be enumerated, or

quantified if quantitative PCR is used. Thus, at the detection level the abundance of the organism allows consideration or elimination of association with an infection. Studies have already demonstrated that the clinical impact was highly associated with the dose -challenge (Collins et al., 2007; Paradis et al., 2012).

The number of enteropathogen involved in intestinal infection exceeds 35 individual species in both pigs and cattle and molecular techniques used for the diagnosis of specific targeted pathogens should take in consideration the geographical epidemiology, the health history of the animal and the general health of the herd. Furthermore, in the context of epidemio-survelliance, repeated sampling is essential for the determination of the implication of the causality of a specific pathogen in the disease.

1. 7. Detection techniques of enteropathogens

1. 7. 1. Conventional techniques

Detection techniques using culture and isolation have become less popular since the 90s being increasingly replaced by molecular techniques. Bacterial detection using culture requires isolation from a sample, and frequently involves a pre-enrichment stage prior to growth in a specific selective medium. This is followed by biochemical or serological tests with the whole process requiring several days for a full identification (Cunningham et al., 2010; Shinde et al., 2012). In addition, some microorganisms such as *Brachyspira* species are not easily cultured (Naresh et al., 2009; Hampson et al., 2015).

The identification of viruses may require their visualisation by electron microscopy involving a specialist facility. In addition, their culture and growth *in vitro* need more exacting protocols. Virus isolation lacks optimal sensitivity or rapidity for consistency in identification (Radwan et al., 1995).

Conventional techniques for protozoan detection are based mainly on microscopic examination. These methods are laborious and require skilled technicians. In addition, enrichment and selective isolation stages can lead to a loss of cysts and oocysts, with a subsequent reduced sensitivity (Rochelle et al., 1997; Wang et al., 2004).

Serological assays including ELISA are both sensitive and specific (Blanchard et al., 2003; Lazcka et al., 2007). However, they monitor the presence of antibodies to specific pathogens and thus reflect exposure to a pathogen and not necessarily the presence of the pathogen itself. As with all tests for infectious diseases in livestock they are used as herd or flock tests with the unit of surveillance being the animal group.

The disadvantages of conventional methods have given impetus to the more rapid molecular approaches to diagnosis and identification. Nucleic acid-based techniques have several advantages including low detection limits, specific organism detection and rapid results (Mothershed and Whitney, 2006).

1. 7. 2. Molecular techniques

The causative association between pathogens and clinical enteritis is aided by the likely presence of high numbers of the pathogen. Conversely sub-clinically infected animals may excrete bacteria intermittently and in low numbers

(House et al., 1993), and in this situation, quantification may only be possible when molecular detection techniques are used.

1. 7. 2. 1. PCR-based techniques

Molecular diagnostics, in particular polymerase chain reaction (PCR)-based tests have become very common in pathogen diagnosis. The PCR method consists of a first step of heat denaturation of DNA, which may be extracted and purified, followed by an annealing of specific primers to a target sequence and an extension phase carried out with a thermostable polymerisation enzyme. These different phases differ in duration and temperature. Each new double stranded DNA acts as a target for a new cycle and amplification is thus exponential. The presence of the amplified sequence is subsequently detected by gel electrophoresis (Xu and Larzul, 1991). PCR is less prone to produce false positives as it detects the organism by amplifying the target rather than the signal (Shinde et al., 2012). However, a false positive is possible resulting from contamination of the sample during collection or in the laboratory (Lappin, 2009).

For microbe detection, PCR offers good specificity, sensitivity, rapidity, accuracy and the capacity to detect small amounts of target nucleic acid in a sample (Toze, 1999), which renders this technique the most widely used in both research and diagnostic laboratories for pathogen detection (Lisby, 1998; Mothershed and Whitney, 2006; Procop, 2007). Although PCR is very sensitive, detection of pathogens in faeces, the basis of this thesis, can be complicated

by the presence of various types of bacteria, including non-pathogens, and potentially inhibitory chemicals (Monteiro et al., 1997).

a. Random and Specific amplification

Random amplification, also called sequence-independent amplification or Random Amplification of Polymorphic DNA (RAPD, pronounced Rapid) uses random primers that can anneal to different sites on the genome and no information of the genome sequence is required. This amplification results in a high number of fragments that are equally dominant. Specific amplification uses a specific pair of primers which targets a sequence of one or more gene(s), the PCR product is amplified exponentionally generating millions of copies. Random amplification requires very small quantities of DNA but also from a sample with a relative purity compared to the specific amplification that is capable to target a specific sequence in a polymixture of genes/genomes. Specific amplification necessitates carefully designed primers based on known sequences. On the other hand, random amplification utilises arbitrary primer sequences. Random amplification can thus lead to less reproductible profiles (Ellsworth et al., 1993).

1. 7. 2. 2. Multiplex PCR

Mixtures of organisms, or multiple sequences of the same pathogen, can be detected by multiplex PCR, which uses more than one pair of primers each targeting individual pathogens.

The advantage of this method over monoplex PCR is that it is well suited to pathological conditions where mixed infections may occur. One frequently

encountered problem with the multiplex PCR assay is an associated reduction in sensitivity due to competition for reagents when multiple templates are amplified in a single reaction (Khamrin et al., 2011). However, the use of a combined three multiplex PCR panel approach for the detection of bacterial, viral and parasitic pathogens in human diarrhoea allowed McAuliffe et al. (2013) to increase the frequency of detection of gastro-enteric pathogens from 18% to 30%.

1. 7. 2. 3. Real-time PCR (RT-PCR) or quantitative PCR (qPCR)

Real-time PCR offers many advantages compared to conventional PCR such as increased sensitivity and rapidity, broader dynamic range and, most importantly, elimination of post amplification handling steps (Smith et al., 2004). In fact, the amplification can be followed in real time offering the elimination of the gel electrophoresis step in a conventional PCR technique, and reducing possible contamination of sample (Monis and Giglio, 2006). Moreover, qPCR is considered as the golden standard method for gene expression assays (Derveaux et al., 2010). The ability to quantify the amount of pathogen nucleic acid in a sample is a huge advantage, especially when mixed infections might be involved to identify the main pathogen.

The most used chemistries in RT-PCR are SYBR Green I (Becker et al., 1996) and probe-based assay, particularly Taqman, probes (Heid et al., 1996), defining detection of binding dyes to the amplified product, or hydrolysed/hybridised probe respectively (Navarro et al., 2015).

The SYBR Green assay principle is based on the use of fluorescent dyes; a specific intercalating dye has little or no fluorescence when free in solution but produces a large quantum yield increase in fluorescence when bound to dsDNA and exposed to the appropriate wavelength of light (Monis and Giglio, 2006). The nucleic acid-specific dye attaches itself to the targeted amplicon generating fluorescence with a proportional intensity to the amount of amplified product (Cady et al., 2005). Exponential quantification is detected from standards with defined numbers of copies of the target fragment (Saunders, 2004). Amplification is thus verified by melting curve analysis, which is analogous to the detection of a band by conventional gel electrophoresis (Monis and Giglio, 2006).

The fluorophore-labelled probe assay is based on the use of two types of fluorophores, reporter and quencher respectively, attached at the 5' and 3' extremities, with the reporter non-fluorescent as long as it stays in a specific distance from the quencher. The probe anneals to the target sequence between the two primers and is cleaved by the polymerase during the extension. The hydrolysis of the probe separates the reporter from the quencher and leads to the release of fluorescence from the reporter.

Detection of amplified targets through emitted fluorescence is realised by a system designed for signal detection in the RT thermal cycler. RT PCR allows the visualization of the instantly quantification of the PCR products.

Several studies have investigated the capability of multiplex real time PCR and singleplex real-time PCR assays to detect mixed enteric virus infections, and

have shown good sensitivity and specificity (Chen et al., 2006; Thao et al., 2010). However, these methods are limited with respect to the number of target pathogens that can be effectively detected (Piao et al., 2012). There is a considerable potential for cross-hybridisation or competition among the multiple primer sets used, resulting in poor PCR amplification and/or the generation of non-specific products (Elnifro et al., 2000). A limitation of this technique is the number of probes used, because each locus must be specifically targeted with a probe that fluoresces at a unique wave-length to differentiate the several amplicons in the same reaction. It is worth noting that current RT thermal cyclers allow a maximum of only six distinct wave-length detection channels (Jex et al., 2012). An additional disadvantage is the cost of the technique in which the price of a single real-time PCR reaction, including DNA extraction, can be more than three times the cost of conventional PCR (Mothershed and Whitney, 2006).

A number of molecular techniques have been used in the detection of the different enteropathogens, mainly PCR-based techniques as they are very commonly used and relatively easy to set up (Table 1.1).

1. 7. 2. 4. LAMP (Loop-Mediated Isothermal Amplification)

LAMP is a relatively recent molecular technique developed in 2000. The method employs four sets of primers that amplify six distinct regions of the target DNA. This technique is highly specific and sensitive as it permits selective recognition of six sequences among a high background of nucleic acid under isothermal conditions. Many authors agree that LAMP technology is less

expensive, rapid and does not need a thermal cycler to carry out the amplification as a simple water bath suffices for the incubation. Its simplicity and the fact that it is equipment-free makes it an ideal method for resource-poor countries with Njiru (2012) highlighting that the LAMP platform can be developed into a realistic point of care format.

LAMP has been utilised in the clinical diagnosis of infectious diseases (Mori and Notomi, 2009) in general and enteric infection in particular. Several authors used LAMP to identify the presence of diverse enteric viruses and bacteria in cattle and pigs (Chen et al., 2010; Ren et al., 2011; Luo et al., 2012; Yin et al., 2012).

Table 1.1. Molecular detection of enteropathogens

Pathogen	Method	Target gene	Reference
Bovine coronavirus	Real-time PCR	Membrane (M)	Decaro et al. (2008b)
	One step RT-PCR	RdRp	Zlateva et al. (2011)
PEDV	PCR	Membrane (M)	Chae et al. (2000)
	PCR	ORF3	Park et al. (2011)
	PCR followed by sequencing	Spike (S): Sequence insertion/mutation- Highly virulent PEDV strains	Li et al. (2012)
	RT-LAMP	Nucleocapsid (N)	Ren and Li (2011)
TGEV	Real-time RT-PCR	Spike (S)	Vemulapalli et al. (2009)
	Multiplex Real-time RT-PCR	Nucleocapsid (N)	Kim et al. (2007)
	LAMP	Nucleocapsid (N)	Chen et al. (2010)
Bovine torovirus	RT-PCR	Spike (S)	Hoet et al. (2003)
	Nested PCR	Spike (S)	Kirisawa et al. (2007)
Bovine and Porcine torovoirus	RT-PCR/ Real-time PCR	Membrane (M)	Hosmillo et al. (2010)
Porcine torovirus	PCR	Nucleocapsid (N)/ Membrane (M)/ Hemagglutinin (HE)	Pignatelli et al. (2009) Pignatelli et al. (2010a)
	Real-time PCR	Nucleocapsid (N)	Pignatelli et al. (2010b)
Porcine circovirus	Real-time PCR	ORF2 (Capsid)	Brunborg et al. (2004)
	Multiplex PCR	ORF1/ORF2	Ouardani et al. (1999)
	LAMP	ORF2	Zhao et al. (2011)

Continued

Pathogen	Method	Target gene	Reference
Bovine enterovirus	RT-PCR	5'UTR	Ley et al. (2002)
	RT-PCR	VP1	Knowles (2005)
	RT-PCR	3'UTR / 3D (RdRp)	Li et al. (2012)
Porcine enterovirus	RT-PCR	5'UTR	Zell et al. (2000)
	RT-PCR	VP1	La Rosa et al. (2006)
	RT-PCR	3D (RdRp)	Koonin and Dolja (1993)
	RT-PCR	N terminus of the capsid protein VP2	Kaku et al. (2001)
	RT-PCR /Nested RT-PCR	5'UTR	Beld et al. (2004) Van Dung et al. (2014)
Bovine kobuvirus	RT-PCR	ORF1/ORF2 junction	Mauroy et al. (2009b)
	PCR	3D (RdRp)[a]	Park et al. (2011b)
	5'/3' RACE/ RT-PCR	3D (RdRp) and 3'UTR region	Reuter and Egyed (2009)
Porcine kobuvirus	RT-PCR	3D (RdRp)	Reuter et al. (2009)
	RT-PCR / Sequencing	3Cpro/3D RdRp	Reuter et al. (2008)
	RT-PCR	VP1	Reuter et al. (2009)
	Real-time RT-PCR	3D (RdRp)	Zhu et al. (2016)
	RT-LAMP	3D (RdRp)	Li et al. (2014)
Sapelovirus	RT-PCR	5'UTR	Lan et al. (2011)
	RT-PCR / Sequencing	VP1	Cano-Gómez et al. (2013)
Teschovirus	RT-PCR	5'UTR	La Rosa et al. (2006)

Continued

Pathogen	Method	Target gene	Reference
Teschovirus	Nested RT-PCR	5'UTR	Chiu et al. (2012)
	RT-PCR	VP3/VP1[b]	Chiu et al. (2012)
Porcine norovirus	RT-PCR	RdRp	Jiang et al. (1999)
	RT-PCR	RdRp – Human Norovirus	Mauroy et al. (2008)
Bovine norovirus	RT-PCR	RdRp	Smiley et al. (2003b)
	RT-PCR/Nested PCR	RdRp	Park et al. (2007)
Porcine sapovirus	RT-PCR	RdRp	Jiang et al. (1999)
		RdRp	Reuter et al. (2010b)
		Capsid	Kim et al., (2006)
Nebovirus	RT-PCR/Nested PCR	RdRp	Smiley et al. (2003b)/ Park et al. (2008)
	RT-PCR	Capsid	Han et al. (2004)
Bovine rotavirus A	Real-time RT-PCR	NSP3/Segment 7	Otto et al. (2015)
	Real-time RT-PCR	NSP3/Segment 7[c]	Pang et al. (2004)
	Real-time RT-PCR	VP7/ Segment 9	Fukai et al. (1999)
	RT-LAMP	VP6/ Segment 6	Xie et al. (2012)
Bovine rotavirus B	Real-time RT-PCR	NSP5/Segment 11	Otto et al. (2015)
Bovine rotavirus C	Real-time RT-PCR	VP6/ Segment 6	Gouvea et al. (1991)/ Otto et al. (2015)
	Real-time RT-PCR	VP7/Segment 9 [d]	Logan et al. (2006)
Porcine rotavirus A	RT-PCR	VP6/ Segment 6	Matthijnssens et al (2008)
	RT-PCR	VP4/	Gentsch et al. (1992)

Continued

Pathogen	Method	Target gene	Reference
Porcine rotavirus A	RT-PCR	VP7/Segment 9	Das et al. (1994)
Porcine rotavirus B	Real-time RT-PCR	VP6/ Segment 6	Marthaler et al. (2014b)
	Real-time RT-PCR	VP7/Segment 9	Marthaler et al. (2012)
Porcine rotavirus C	Real-time RT-PCR	VP6/ Segment 6	Jeong et al. (2009)
	RT-PCR	VP6/ Segment 6[e]	Sánchez-Fauquier et al. (2003)
	RT-PCR	VP7/ Segment 9	Rahman et al. (2005)
Porcine rotavirus A/C	Multiplex Real-time RT-PCR	VP6/ Segment 6	Marthaler et al. (2014a)
Porcine orthoreovirus	RT-PCR	L1	Decaro et al. (2005)
	RT-PCR	S1	Narayanappa et al. (2015)
Bovine parvovirus	PCR/ LAMP	VP2	Luo et al. (2012)
Porcine parvovirus	PCR	VP1/VP2	Pinheiro de Oliveira et al. (2016)
	Real-time PCR	ORF3	Gava et al. (2015)
	Real-time PCR	ORF2	Xiao et al. (2013)
Porcine bocavirus	Real-time PCR	NP1	Li et al. (2011)
	LAMP	VP1/VP2	Li et al. (2012)
	Duplex Nano PCR	NS1	Luo et al. (2015)
BVDV	Real-time RT-PCR	5'UTR	Letellier and Kerkhofs (2003)
	RT-PCR	N^{pro}	Becher et al. (1997)
	RT-PCR 2 steps	E2	van Rijn et al. (1997)
	Multiplex Real-time RT-PCR[f]	5'UTR	Baxi et al. (2006)

Continued

Pathogen	Method	Target gene	Reference
Bacteroides fragilis	Real-time PCR	recA	Stappers et al. (2016)
	Real-time PCR	gyrB	Lee and Lee (2010)
	Nested PCR	bft	Shetab et al. (1998)
Campylobacter jejuni	PCR	mapA	Stucki et al. (1995)
Campylobacter coli	PCR	ceuE	Gonzalez et al. (1997)/ Denis et al. (1999)
C. jejuni/*C. coli*	Multiplex PCR	C.jejuni hipO and 23S rRNA/ C.coli glyA	Wang et al. (2002)
Clostridium spp.	PCR	16S rDNA	Kikuchi et al. (2002)
Clostridium difficile	PCR	cdtB	Gumerlock et al. (1993)
	PCR	cdtA	Tang et al. (1994)
	PCR	cdtA , cdtB	Kato et al. (1991)
	Multiplex PCR	16SrDNA, tcdA ,tcdB , cdtA , cdtB	Persson et al. (2008)
Clostridium perfringens	Multiplex PCR	cpa , cpb, cpe, iA ,etx, ibp	Meer and Songer (1997)
	Multiplex Real-time PCR	cpa , cpb, cpe, iA ,etx, iap , cpb2	Albini et al. (2008)
	Real-time PCR	16S rDNA	Wise and Siragusa (2005)
Escherichia coli ETEC	PCR	sta	Valat et al. (2014)
Escherichia coli STEC	PCR	stx1, stx2	Valat et al. (2014)
Escherichia coli EAEC	Multiplex PCR	aat, aaiA, astA	Jenkins et al. (2006)
E. coli STEC / EPEC	PCR	eae	Valat et al. (2014)

Continued

Pathogen	**Method**	**Target gene**	**Reference**
E. coli EAEC/ETEC	PCR	astA	Valat et al. (2014)
E.coli ETEC/EHEC/EPEC/EIEC/EAEC	Multiplex PCR	*eltB, estA/ vt1, vt2, eaeA/ eaeA*, *bfpA/ ial/* pCVD432-harboring strain	Nguyen et al. (2005)
E.coli EAEC/ETEC/EPEC/STEC/EIEC/DAEC	Multiplex Real-time PCR	*aggR*/*stla*, *stlb*, *lt/ eaeA*/*eaeA*, *stx1*, *stx2/ ipaH/ daaD*	Guion et al. (2008)
Salmonella enterica	PCR	*invA, invE*	Stone et al. (1994)
Salmonella Typhimurium	Multiplex PCR	*oafA , fliC , fljB*	He et al. (2016)
Salmonella Typhimurium	PCR	*fliA-B, fljB, fliC, mdh*	Bugarel et al. (2012)
Salmonella Enteritidis	Multiplex PCR	*invA, fliC-k, fliC-l, fliC-r, sdf*	Shimizu et al. (2014)
S. Typhimurium/Enteritidis	Multiplex Real-time PCR	*safA, fliA,fljB, hin-iroB, IAC* (pUC18/19)	Maurischat et al. (2015)
Salmonella Dublin	Multiplex PCR	*invA,* SeD_A1118, SeD_A2283	Zhai et al. (2014)
Non-typhoidal invasive *Salmonella enterica*	Multiplex PCR	16S rRNA, *fliC-gp*, *fliC-i*, *fliC-z4*, *z23, sdfI*	Tennant et al. (2010)
Salmonella Choleraesuis	LAMP	*ofjliC*	Yin et al. (2012)
Salmonella Choleraesuis	PCR	*flinC*	Chiu et al. (2005)
Yersinia enterocolitica (among other enteropathogens)	Multiplex PCR	*Yst*	Gómez-Duarte et al. (2009)
Yersinia enterocolitica	PCR	*yadA*	Bonardi et al. (2007)

Continued

Pathogen	**Method**	**Target gene**	**Reference**
Brachyspira hyodysenteriae / *Brachyspira pilosicoli*	Multiplex PCR	*nox,* Unknown recombinant clone pRED3C6) / *16S rDNA*	Nathues et al. (2007)
Lawsonia intracellularis	PCR	16S rDNA, chromosome fragment	Dittmar et al. (2003)
Lawsonia intracellularis	Real-time PCR	16S rRNA	Richter et al. (2010)
Trichuris suis	PCR	Internal transcribed spacers ITS1-5.8S-ITS2[g]	Cutillas et al. (2007)
Cryptosporidium spp.	PCR	18S rDNA	Xiao et al. (1999)
Giardia spp.	PCR	β-giardin	Cacciò et al. (2002)
Eimeria spp.	Multiplex PCR	Specific internal transcribed spacers (ITS-1)	You (2014)
Isospora suis	Nested PCR	18S rRNA	Joachim et al. (2004)

a: 3D region of kobuviruses including Aichivirus, Bovine kobuvirus and Porcine kobuvirus; b : Teschovirus serotype 1; c: Designed from Human rotavirus A; d: Designed from Human rotavirus C; e: Designed from Human rotavirus C; f: Simultaneous detection of BVDV1 and BVDV2; g: Flanking the 18S and 28S regions

RT-PCR: Reverse Transcription PCR

RT-LAMP: Reverse Transcription Loop-Mediated Isothermal Amplification

1. 7. 3. DNA Microarrays

Microarray technology arose from the principle of the Southern blot which was a detection method based on the transfer of separated bands of DNA or RNA by electrophoresis to a membrane followed by a hybridisation with the corresponding labelled probes (Alwine et al., 1977). In contrast, the microarray probes are immobilized and their targets are labelled and free in the hybridisation solution. After hybridisation, the duplex formed by the probe and its complementary sequence are detected by fluorescence or enzymatic reaction.

A microarray is a collection of probes attached or "spotted" in a pattern/array (Schena et al., 1995). The spotted surface can be a glass slide, coated glass or silicon slide. Probes may be printed robotically and immobilised ideally through a spacer molecule of 40 atoms in length (Shchepinov et al., 1997).

Large numbers (10^6) of oligonucleotides can be arrayed on a surface (Leroy and Raoult, 2010) which can thus provide very extensive coverage because a high number of possible DNA elements can be interrogated in a single experiment and with a single sample (Uttamchandani et al., 2009; Sibley et al., 2012).

Two types of microarrays are generally used, oligonucleotide and PCR product microarrays. Oligonucleotide probe sizes vary from 20 to 70 nucleotides, whereas PCR probes sizes range between 200 and 2000bp (He et al., 2005). PCR probes are more difficult to prepare because of the large number of amplifications required to be performed, in addition to the contamination risk during handling (Nsofor, 2014). The use of oligonucleotide microarrays has

linearly developed because it allows a high density synthesis onto the microarray surface (Schrenzel et al., 2009).

1. 7. 3. 1. Microarray platforms

Different platforms are available commercially with probes contact-spotted, ink-jet deposited or synthesised directly on the substrate (Coppée, 2008) and a range of probe lengths or densities are available, emphasising their technical flexibility. Only the most commonly used platforms will be described in this section. Additionally, the Alere microarray (ArrayTube platform), a less known platform, will be also described as it has been used for virus detection in this project.

a. Agilent

Agilent (http://www.genomics.agilent.com/article.jsp?pageId=2011) manufactures a high-density microarray that employs SurePrint inkjet technology to print the probes onto glass slides, with layers of DNA nucleotides deposited individually based on a digital sequence file. Up to eight microarrays can be spotted on a single slide at the same time. Probes are usually 60mers long. Agilent offers off-the-shelf and custom microarrays. With the built-in application eArray (https://earray.chem.agilent.com/earray/helppages/index.htm), researchers are able to design personalised microarrays, which can involve either one or two-colours.

The different formats of gene expression and custom microarray have a capacity of 8x60K, 8x15K, 4x180K, 4x44K, 2x400K, 2x105K, 1x1M, 1x244K, probes.

b. Affymetrix

Affymetrix (http://www.affymetrix.com/estore/browse/level_one_category_template_one.jsp?category=35796) owns several ready to use genotyping and customisable microarrays, which can accommodate more than 10^6 probes. The GeneChip platform is a quartz wafer of 1.2 cm^2 onto which probes are synthesised using photolithography. This consists of the synthesis of linkers, modified with light-sensitive protecting groups (Fodor et al., 1991; Dalma - Weiszhausz et al., 2006). The probes are short (20-25-mers) and this platform allows the use of only one colour (Miller and Tang, 2009).

c. Illumina

Illumina (http://www.illumina.com/techniques/microarrays.html) microarray technology consists of silica beads assembled in microwells on fibre optic bundles or planar silica slides. Two types of substrates are utilised, the Sentrix Array Matrix (SAM) and the Sentrix BeadChip (Fan et al., 2006a). The SAM platform contains 96 1.4mm fibre optic bundles chemically etched to create a well for a 3µm bead that holds approximately 700k probes (Fan et al., 2006b). The Sentrix BeadChip is a silicon slide that can be used to assay 1 to 16 samples at a time, whereas the SAM is more suitable for very high density applications, it allows the analysis of 96 independent samples.

During fabrication, the beads are self-assembled randomly onto the patterned substrate, which necessitates a decoding system to map the exact location of each bead (Gunderson et al., 2004). Both Illumina platforms allow multi-colour hybridisation.

d. Alere Technologies

Alere (http://alere-technologies.com/en/products/lab-solutions/platforms/arraytube-at.html) manufactures three array formats. The ArrayStrip is formed of eight wells and at the bottom of each is a 4x4 mm chip inserted with 784 probes. The ArrayTube consists of a microcentrifuge tube fitted at the bottom with a 3x3mm chip with 196 features. The Arraytube2 is the most recently developed platform of with a chip of 4x4mm that holds 540 probes.

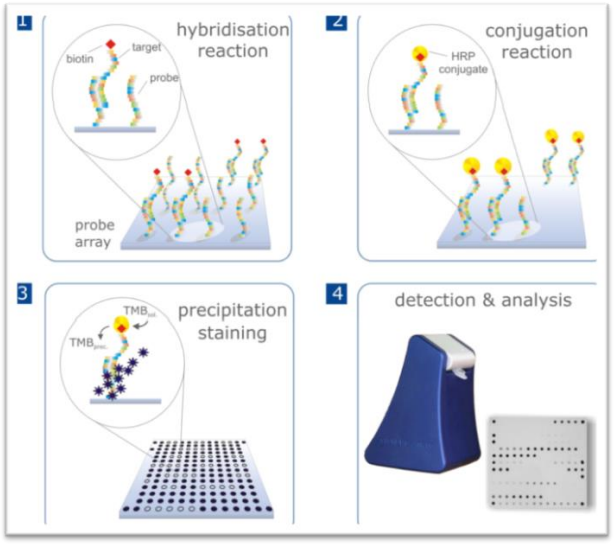
1. 7. 3. 2. Principle

Microarrays are based on the ability of PCR to amplify and label the targeted DNA present in a specimen. This step is followed by hybridisation to the probe matrix of the microarray. An increased signal intensity above the background level results from a successful hybridisation event between the labelled target molecule and the immobilised probe, which is then quantified using an array scanner (Sibley et al., 2012).

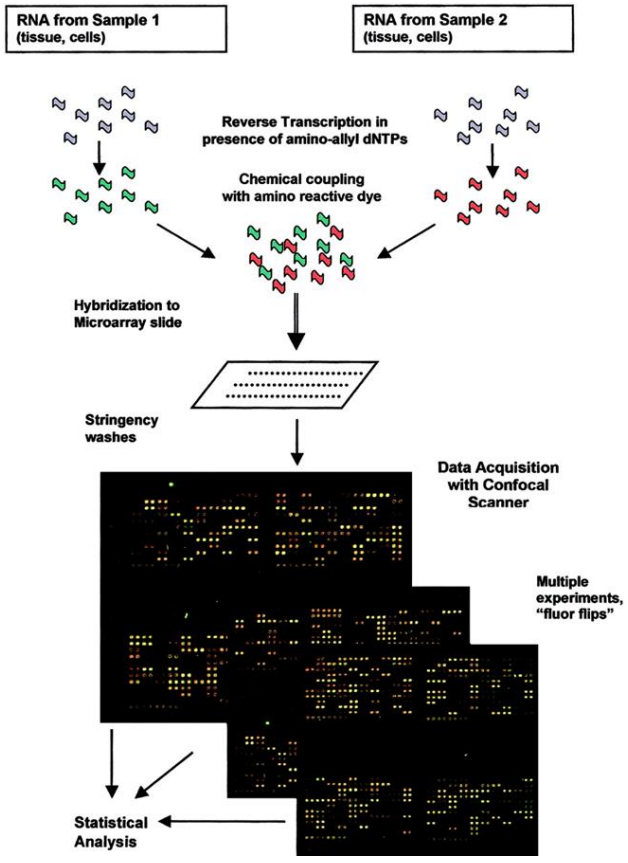
Hybridisations can be performed using one or two fluorescent dyes of different colours; two-colour arrays can be hybridised simultaneously with two different samples, each labelled with a different fluorochrome (Shalon et al., 1996) whereas in one colour hybridisation the samples are hybridised separately on

a single array (Leroy and Raoult, 2010). Enzymatic alternatives have been used for labelling, such as biotin that allows detection through a secondary label (peroxidase streptavidin). Biotin–streptavidin colorimetric schemes represent an inexpensive labelling technique compared with fluorescent dyes (Alexandre et al., 2001), although indirect detection methods add additional steps to the detection process (Call et al., 2003).

The data subsequently generated may be analysed with a variety of bioinformatic algorithms (Zhang et al., 2009; Kaewpongsri et al., 2010; Loewe and Nelson, 2011) . Hybridised target-probe complexes appears as spots in the final image where each spot is usually 100–200 µm in size and located within 200–500 µm of each other (Call et al., 2003). Figure 1.1 illustrates the principle of the hybridisation assay in biotin-labelled (A) and fluorescently-labelled (B) assays.



(A)



(B)

Figure 1.1. Schematic representation of the principle of hybridisation assays. (A): Alere platform, sample is labelled with biotin and hybridised to the platform (Adapted from Alere Technologies) and (B): Showing two samples labelled with fluorescent and hybridised onto the same platform (Arcellana-Panlilio and Robbins, 2002)

1. 7. 3. 3. Microarrays in enteric infection diagnosis

Microarrays have been used to identify, characterise and to genotype various types of microorganisms including bacteria, viruses, parasites, and fungi (Bryant et al., 2004). This has assisted in the discovery of previously unknown pathogens such as human rhinovirus (Kistler et al., 2007), human cardiovirus (Chiu et al., 2008), pandemic influenza H1N1 (Greninger et al., 2010), Titi monkey adenovirus (Chen et al., 2011) and human polyomavirus (Yu et al., 2012).

Most microarray-based detection investigations have targeted pathogens infecting children with diarrhoea (Martínez et al., 2015), the human microbiome (Ballarini et al., 2013) and clinically important viral and bacterial enteric pathogens in human and environmental samples (Sun et al., 2010). Authors have also directed their efforts into detecting virulence and antimicrobial resistance genes in *Salmonella* serovars and *E. coli* (Chen et al., 2005; Gorski et al., 2011) with additional other interests to understand the gene expression of pathogens(Danckert et al., 2014) or to determine a source of faecal contamination (Dubinsky et al., 2012).

In animals, few studies have described multiple detection and identification of viral, bacterial and parasitic enteropathogens in cattle and pigs using microarrays. Recent studies have developed microarrays to detect only one to two specific enteropathogens, for example the identification of porcine circovirus (PCV) genotypes in pigs (Jiang et al., 2010), *E. coli* serogroups associated with bovine septicaemia and diarrhoeal disease (Liu et al., 2010) or

identification of intracellular signalling molecules in cattle and pigs after infections with one to two pathogens (Werling et al., 2005; Jacobson et al., 2011).

The multi-infectious nature of many cases of enteritis (Parra et al., 2011; Cho et al., 2013; Song et al., 2015) with confirmed co-infections with viruses, bacteria and parasites suggest that microarrays could fulfil the need for a diagnostic tool that can specifically detect simultaneously the different pathogens. This will require meticulous probe design, coupled with an appropriate DNA/RNA extraction and amplification strategy.

The most recent research related to microarray technology are two studies on parallel detection of 33 enteropathogenic bacteria and seven enteropathogenic viruses in humans (Donatin et al., 2013) and 100 viral species infecting humans and other animals (Martínez et al., 2015). The first study confirmed the multidetection of enteric pathogen in human stools, the second showed a good performance of the microarray albeit with low sensitivity for detecting human adenovirus and human enterovirus but it did fail to detect multiple infections. As far as is known, the use of DNA microarrays for the simultaneous detection of all possible enteric pathogens infecting cattle and pigs has not been reported.

1. 7. 4. Sequencing

Sanger sequencing is now considered as the traditional method of sequencing, which is based on the use of dideoxynucleotides labelled with fluorescent dyes which terminate the polymerisation process, thus termed as the chain-

terminator method (Sanger and Coulson, 1975). The sequence is then determined based on the identity of the last terminator base after separation of the fragments by size (Hert et al., 2008).

1. 7. 5. Next generation sequencing

Next generation sequencing (NGS) also termed as deep or high-throughput or second generation sequencing comprises different platforms that include pyrosequencing, sequencing by ligation, ion semiconductor sequencing and sequence by synthesis. It is the most commonly used method and will be briefly described below.

As the technology progresses aiming to overcome limitations mainly due to the reliability and the cost, third generation sequencing technologies including nanopore sequencing through fluorescent resonant energy transfer have been cited (Diaz-Sanchez et al., 2013).

A large range of next generation sequencing platforms, mainly manufactured by Illumina and Life Technologies are commercially available (Loman et al., 2012). The NGS illumina technique consists of two main steps, library construction that involves the extraction and purification of genomic DNA from a sample and template amplification and sequencing, which consists of the fragmentation of the extracted genome into random overlapping fragments, generally of 150 to 800 bp (also called tagmentation). Adaptors are then ligated to the obtained fragments. The sequencing step starts first by carrying out a bridge PCR, comprising the synthesis of a complementary strand of the template and the adaptor which possesses a complementary structure linked

to the surface of the platform. Amplified products are bent and immobilised forming molecular bridges, hence the name. End adaptors are then added after a second fragmentation. Cycles of sequencing are then performed through the joins which are important to determine the location of the sequence in the genome and for the assembly (Loman et al., 2012).

NGS has been used to a moderate extent in the diagnosis and monitoring of infectious diseases (Lefterova et al., 2015). According to Fournier et al. (2014), 38,000 bacterial and 5,000 viral genomes have been sequenced using NGS with a significant number of human pathogens. With the advent of metagenomics, where a mixture of microrganism genomes are sequenced (Lam et al., 2012), whole-genome sequencing (WGS) has been adopted by several researchers as it reveals the sequence of the entire strain genome and can uncover specific genetic markers, molecular epidemiology and phylogenetic relationships between strains.

Setting up a microbial sequencing facility is still prohibitively expensive, a set of instruments is necessary with a cost ranging from $495,000 and $690,000. In terms of cost of a single experiment cost, it varies between $6,300 and $15,000/100Gb, with Illumina being the most affordable (Hert et al., 2008; Liu et al., 2012). NGS techniques were applied to different fields of biological interests, beside the WGS, other techniques related to genomics, transcriptomics and epigenomics have emerged these recent years. They include *de novo* sequencing, resequencing, HiC, Chip-Seq and RNA-Seq (Nowrousian, 2010). Commercially available platforms besides Sanger

sequencing which principle is based on the dideoxy chain termination, Roche 454 based on pyrosequencing, HiSeq that relies on the sequencing by synthesis and SOLiD that uses sequencing by ligation (Liu et al., 2012).

NGS is an already established technology, its attractiveness resides in the level of biological meaning of results from processing a sample, however despite the per-base cost of a sequencing run has been reduced, the cost and maintenance of NGS equipment is still unaffordable for modest projects. Alongside the cost of NGS equipment, one of the drawbacks of NGS is also the enormous amount of data generated in one single experiment which requires bioinformatics skills, besides the data storage facility that accommodates intensive processing of samples.

However more reliable than microarray, sequencing lacks innovation and intrinsic development potential unless platform-related changes/ improvements are undertaken. Microarrays are nevertheless extremely customisable and adaptable to the need of the researcher, besides the fact that it is a cost-effective platform compared to NGS. Table 1.2 highlights the general costs of the different sequencers and the per-base cost run in the different platforms.

Depending on the academic and non academic pricing, the cost of sequencing per sample can vary from $1550 to $4525 and when using the Illumina NGS 500, the library preparation cost is not included. Affymetrix microarray for gene expression using the most commonly used array varies between $335 and $695, the cost including arrays, reagents, processing, and basic gene-level data

analysis (http://www.bumc.bu.edu/microarray/pricing/). The costs for Agilent DNA microarray are lower, starting at $250 per sample, although, however, the cost of sample preparation and labelling, hybridisation and scanning are charged separately.

Regarding the cost of the most commonly used molecular technique in the detection of genes (PCR), an extensive study that included the sample preparation and reagents, the use of the equipment and the labour of technicians for the detection of mycobacterial infections in human estimated that the cost varies between $5-10 per sample (Scherer et al., 2009).

Table 1.2. Components and costs of sequences (Liu et al., 2012)

Sequencers	**454 GS FLX**	**HiSeq 2000**	**SOLiDv4**	**3730xl**
Instrument price	Instrument $500,000, $7000 per run	Instrument $690,000, $6000/(30x) human genome	Instrument $495,000, $15,000/100 Gb	Instrument $95,000, about $4 per 800 bp reaction
CPU	2* Intel Xeon X5675	2* Intel Xeon X5560	8* processor 2.0 GHz	Pentium IV 3.0 GHz
Memory	48 GB	48 GB	16 GB	1 GB
Hard disk	1.1 TB	3 TB	10 TB	280 GB
Automation in library preparation	Yes	Yes	Yes	No
Other required device	REM e system	cBot system	EZ beads system	No
Cost/million bases	$10	$0.07	$0.13	$2400

1. 7. 5. 1. The use of NGS for enteric pathogen identification

Enteric pathogens have been detected and identified by NGS mainly in the human gut microbiome that takes in account the entire community dynamics of the intestinal flora. Indeed, the qualitative and quantitative study of the gut flora has demonstrated a close relationship to the general health of individuals (Riley et al., 2013). Moreover, quantitative metagenomics allows the diagnostic of disease and is able to identify predisposed individual which can develop disease (Ehrlich, 2016). The intestinal microbiota was studied in pigs using high throughput sequencing for the identification of the V1- V3 hypervariable region of the 16S rRNA genes which allowed determination of the core group of microrganisms and their relative proportions (Park et al., 2014; Pajarillo et al., 2015). Others have reported the identification by NGS of a newly recognised virus (tentatively named Kırklareli virus) belonging to the *Calciviridae* family that was isolated from calves during an outbreak of enteritis (Alkan et al., 2015). Another recent study employed genomic sequencing to describe a novel porcine deltacoronavirus that has caused a significant recent economic loss in the swine industry (Ma et al., 2015).

1. 8. Aims and objectives

The main aims of this project were to design and develop a detection tool using microarray technology for the simultaneous detection of different enteric pathogens infecting cattle and pigs and to begin to evaluate the contribution of co-existing pathogens in diarrhoeic samples from the UK and Algeria.

The objectives were:

- Probe design using different software.
- *In silico* hybridisation predictions of probes with targets using nearest neighbour model (Dimitrov and Zuker, 2004; SantaLucia and Hicks, 2004; Markham and Zuker, 2005).
- Assess the sensitivity and specificity of two microarray platforms (Alere – ArrayTube and Agilent)
- Assessment and evaluation of the arrays with cattle and pig clinical samples from the UK and Algeria.

The Agilent and Alere platforms were chosen because of the availability of the hybridisation equipment and the detection systems for both platforms in this laboratory. Other microarray platforms are commercially available, such as Affymetrix and Illumina, both offering customized and pre-designed microarrays. Affymetrix produces GeneChip arrays to identify pathogenic agents and has recently developed an Axiom Microbiome array for the microbial profiling of over 12,000 species. Equally, Illumina offers a range of ready-to-use bead microarrays and the possibility to design a genotyping panel adapted to the researchers' needs.

Designing microarray experiments with Alere and Agilent was also a choice based on the technology itself, where long probes were used compared to Affymetrix that uses short probes of 25-mers and Illumina of 50-mers. Long probes of 60-mers represent a fair compromise between specificity and sensitivity. Our choice was also motivated by the costs of the arrays that were reasonably affordable compared to Affymetrix and Illumina.

Initially, probes that were designed for virus identification using one software only (UPS), were spotted in the ArrayTube (Alere). Other software (Picky, Goarray and eArray) were then used to design probes using the same viral genes, but also bacterial and parasitic genes, resulting in thousands of probes that were afterwards spotted on an Agilent platform.

The two platforms differ in terms of labelling chemistry (fluorescent and biotin dyes) and probe density (high and low density) for Agilent and Alere respectively.

Chapter 2. Experimental approach

2. 1. Methodology

A set of enteropathogens infecting cattle and pigs was selected and a gene database constructed accordingly, from which primers and probes were then designed (Discussed in detail in Chapter 3). Two different microarray platforms were constructed; their evaluation involved testing with nucleic acid from reference species, and from clinical faecal samples (Chapter 5 and 6). Also the specificity and sensitivity of the arrays were assessed. Figure 2.1. Illustrates the organogram of the work carried out.

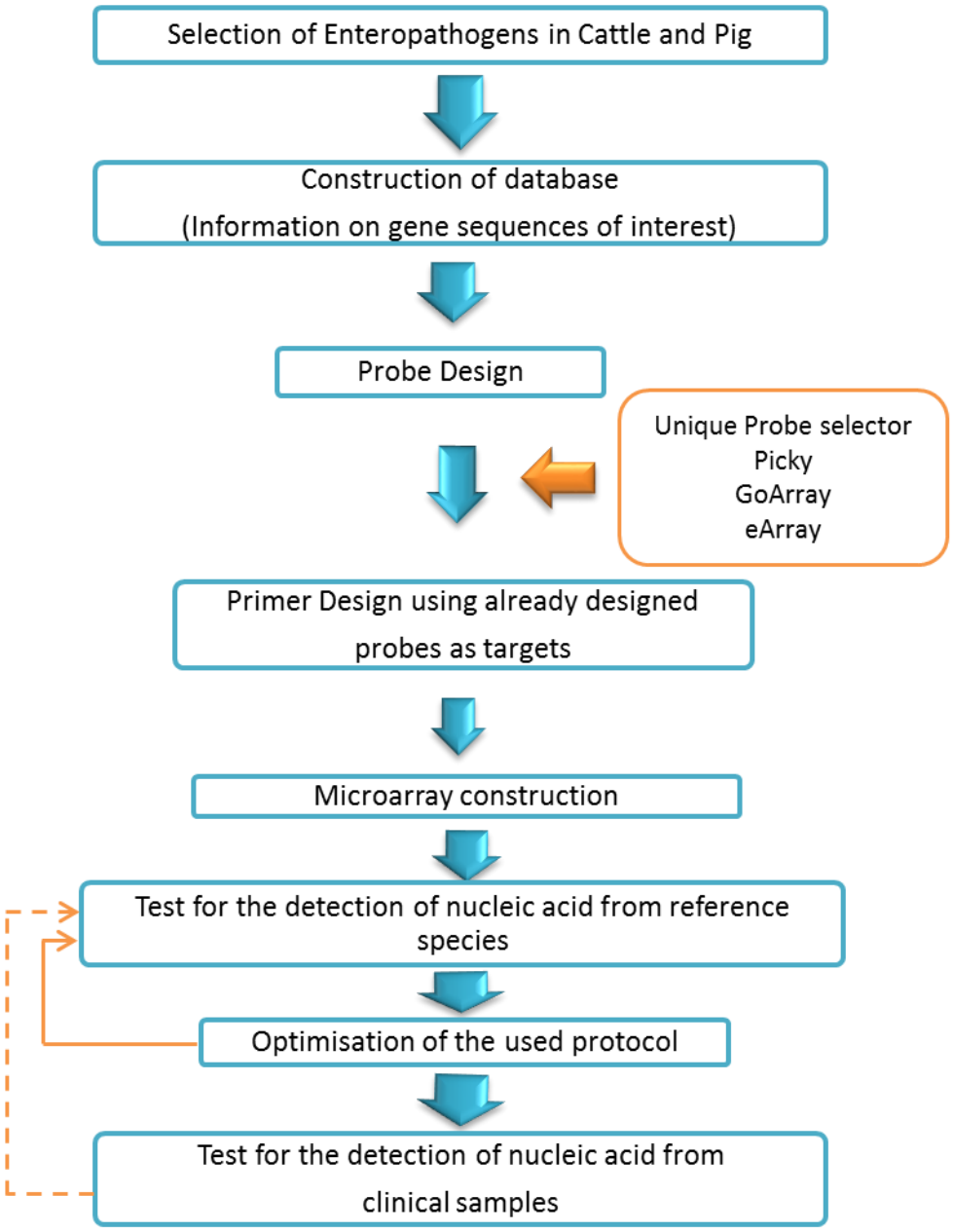


Figure 2.1. Flowchart of the work carried out in the project

2. 2. Samples

2. 2. 1. Reference samples

Based on published research for the pathogens selected, several authors in the UK and other European countries were contacted requesting nucleic acids from the relevant pathogens of interest. Table 2.1. illustrates the list of available and donated species / nucleic acid and reportedly positive faecal samples and their suppliers.

Table 2.1. Donated reference samples

Microorganism	Sample type	Supplier
Clostridium difficile	DNA	Dr S. Kuehe, Biomedical Science - Nottingham University- UK
Salmonella Typhimurium	Bacteria	Prof P. Barrow, School of Veterinary Medicine and Science, The University of Nottingham - UK
Salmonella Enteritidis	Bacteria	
Escherichia coli F4 (K88)	Bacteria	
Escherichia coli F5 (K99)	Bacteria	
Campylobacter jejuni	DNA	Dr S. Houton, School of Biosciences, The University of Nottingham - UK
Brachyspira pilosicoli	DNA	Prof. R. LaRagione School of Veterinary Medicine, University of Surrey -UK
Cryptosporidium spp.	DNA	Dr A. Zintl, School of Veterinary Medicine, University College Dublin - Ireland
Eimeria acervulina	DNA	Dr D. Blake, Royal Veterinary College, London- UK
Porcine torovirus	Positive faecal sample	D. Rodriguez, Departamento de Biologia Molecular y Celular, Universidad Autónoma de Madrid, Madrid, Spain.
Bovine kobuvirus	Positive faecal sample	Dr A. Mauroy, Faculty of Veterinary Medicine, University of Liège- Belgium

Continued

Microorganism	Sample type	Supplier
Bovine norovirus (Newbury2)	RNA	Dr F. D'Mello, Royal Veterinary College, London- UK
Bovine nebovirus (Newbury1)	RNA	
Porcine rotavirus	Positive faecal sample	Dr M. Le Bon, Food Science - Nottingham University - UK
Porcine rotavirus A	cDNA	R. Chandler, Food Science - Nottingham University - UK
Porcine parvovirus 1-2-3-4	DNA	Dr C. Attila, Faculty of Veterinary Science, Budapest - Hungary
Porcine bocavirus 1-2	DNA	Dr C. Attila, Faculty of Veterinary Science, Budapest - Hungary
Porcine bocavirus 3-4-5	DNA	Dr C. Attila, Faculty of Veterinary Science, Budapest - Hungary
Porcine circovirus 2	DNA	Dr C. Attila, Faculty of Veterinary Science, Budapest - Hungary
Porcine epidemic diarrhoea virus	RNA	Prof L. Enjuanes, Centro Nacional de Biotecnologia – Spain
Transmissible gastroenteritis virus	RNA	Dr A. Abu-Median, School of Veterinary Medicine and Science, Nottingham – UK, courtesy of L. Enjuanes
Bovine viral diarrhoea virus CP	Medium supernatant	Dr A. Abu-Median, Courtesy of Dr M. Iqbal, The Pirbright Institute, Compton – UK
Bovine viral diarrhea virus NCP	Medium supernatant	
Bunyamwera virus (B1), Schmallenberg virus (S) Equine influenza A virus (H3N8).	Medium supernatant	Dr J. Daly, School of Veterinary Medicine and Science, Nottingham – UK

2. 2. 2. Clinical samples

Faecal samples from pigs were donated by Dr M. Le Bon, University of Nottingham (Appendix I – I.1). These samples were collected as part of a study involving a trial on the effect of probiotics on the incidence of some

enteropathogens in France. Bovine clinical samples were mostly collected from diarrhoeic calves of less than three months of age and a few cows from farms in the central north of Algeria. No information about breed, nutritional status, hygiene status of the calves or farm management was available. Appendix I.1 and I.2 show pig and cattle/camel/goat samples with the corresponding amount of extracted DNA and RNA (ng/µl).

2. 3. Nucleic acid extractions

Depending on the biological material, different commercial kits were employed to extract nucleic acid. For faecal material, RTP®Pathogen Kit (Stratec Molecular) and QIAamp® Stool Mini Kit (Qiagen) were utilised, while purification of DNA from pig intestinal tissue and RNA from BVDV medium supernatant and other virus cell culture supernatants were performed with a DNA Mini Kit (Qiagen), Viral RNA Mini Kit (Qiagen) and RNeasy Mini Kit (Qiagen) respectively. The QIAamp® UCP Pathogen Mini Kit (Qiagen) was used to purify DNA from bacterial broth cultures. Unless otherwise stated, all centrifugation steps were carried out at room temperature using a benchtop centrifuge. High temperature incubations and vortexing were carried out using a thermomixer (BioShake iQ, Quatifoil Instruments GmbH). DNA amplification was performed on an Applied BioSystems 2720 thermal cycler (Life Technologies). All nucleic acid extractions were performed using reagents that come with the kits, also, lysis tubes, columns and buffers come with the kit.

2. 4. Microarray hybridisation

Nucleic acid extracted from reference and cattle and pig clinical samples were amplified prior to hybridisation onto the Alere and Agilent platforms. Two amplification strategies were adopted, random amplification (5.2.4.1 and 5.2.4.2 - Chapter 5) and specific amplification (4.2.7 - Chapter 4). All randomly-amplified samples were biotin- labelled according to the protocol described in 5.2.4.3 – Chapter 5, whereas sequence-specific amplified samples were either biotin- labelled or fluorescently labelled after being subjected to a random amplification and therefore were treated as DNA samples (6.2.3 – Chapter 6). Labelling with biotin was done with samples that were hybridised on the Alere platform (Chapter 5), and samples hybridised on Agilent platform were labelled with fluorescent dyes, Cy-3 and Cy-5 (Chapter 6). These were used to label two different samples, generally virus/bacteria or porcine sample/bovine sample. The reason for using dual hybridisation of two different samples on the same array was to ensure a maximum number of hybridisations on the Agilent array because of economic reasons, as a limited number of microarrays were available. We appreciate that the use of two different platforms and different labelling chemistries did not permit a strict comparison between the two platforms. Further the Alere platform (low density microarray) does not possess sufficient spot capability compared to the Agilent platform to include all the designed probes which was thus limited to 198 spots.

2. 5. Assessing array specificity

The specificity of the two platforms were assessed using reference strains that are represented in both arrays and strains that are not covered by the microarray for the ArrayTube platform only. Also, clinical samples were hybridised to the arrays; concomitantly, PCR reactions were carried out to confirm the presence of pathogens in the samples.

2. 6. Assessing array sensitivity

Sensitivity analysis involved testing the sensitivity of the two platforms compared to the sensitivity of PCR, quantitative PCR (qPCR) and bacterial culture.

Copy number was calculated using the following formula:

$$\text{Copy number} = \frac{\text{Amount of ds/ss DNA/RNA (ng)} \times 6.022 \times 10^{23}}{\text{Length of ds/ss DNA/RNA (bp)} \times 1 \times 10^{9} \times 630\,(\text{ds})/330\,(\text{ss})}$$

2. 7. Pathogen detection in clinical samples by microarray

Both Alere and Agilent platforms were used to detect viruses, bacteria and parasites in clinical samples from diarrhoeic piglets and calves. As clinical samples were first screened for common enteropathogens by PCR, only a few selected samples that were positive by PCR, showing the presence of one or multiple pathogens, were hybridised on both microarray platforms.

Chapter 3. Probe Design, Software Comparison and Thermodynamics

3. 1. Introduction

Designing probes can involve different strategies. They can be selected from conserved or variable regions on a genome after alignment or they can be designed by an online probe design tool or installed software from a submitted batch of gene sequences. In this study, four different softwares were employed to design probe oligonucleotides: Unique Probe Selector (UPS), Picky, GoArray and eArray. The choice of these software relied on their online availability or their easy access possible through their developers, with the exception of eArray which is a probe design application owned by Agilent. This would allow the comparison of 4 types of probes in terms of rate of detection but also in terms of their suitability/ compatibility of the probes with the platform used (eArray - Agilent probes).

3. 1. 1. Probe design strategies

UPS (http://array.iis.sinica.edu.tw/ups/) selects probes on the basis of the construction of a suffix array, which is a methodical list of strings and substrings that are in the same sequence. This strategy allows the categorisation of the sequences in several words that are compared for their similarity (Manber and Myers, 1993). It carries out different tests to minimise background noise during hybridisation involving several parameters such as GC content, GC clamps, the

duplex stability estimated by nearest neighbour model, secondary structures and low-complexity mask (Chen et al., 2010).

Among the practical positive attributes of UPS, are the use of a non-redundant NCBI nucleotide database (NCBI_NT) as the background reference set, the exclusion of non-target sequences which are >85% similar to the submitted batch, in addition to the exclusion of oligonucleotides that have more than 17 bases of continuous identical fragment to non-target sequences. In addition, these parameters along with a test using BLASTn are *in silico* measures taken to prevent cross-hybridisation when performing the hybridisation step.

Picky software housed in Complex Computation laboratory – Iowa State University web site (https://www.complex.iastate.edu/download/Picky/download.html) uses the same strategy as UPS by establishing suffix trees for the choice of probes. Basically it detects the positions of all suffixes and classifies them in alphabetical order to identify shared regions in the sequence. Thermodynamic estimations use the nearest neighbour model of sequences that have 75% or more identity. Another feature of Picky is assessing the candidate probes for secondary structures and similarity with complementary strands (Chou et al., 2004). Another characteristic is that it displays shared regions among sequences to distinguish them clearly from other highly similar but non-target regions during thermodynamic comparisons (Chou, 2010).

GoArray (http://g2im.u-clermont1.fr/serimour/goarrays.html) employs a completely different approach to select probes (Rimour et al., 2005). Assuming

that short oligonucleotides are more specific than long oligonucleotides, the construction of a long oligonucleotide is based on the selection of two sub-sequences joined by a short random linker of 3-6 bases. The resulting oligonucleotide is a long probe formed by two specific sequences. When hybridised, they lead to the formation of a loop in the target sequence. The algorithm checks the specificity of the two sub-sequences separately with BLAST taking into account that an identical stretch of a minimum of 15 bases, or more than 75% sequence identity with non-target sequence, is considered as non-specific.

eArray (http://www.genomics.agilent.com/en/Custom-Design-Tools/eArray/?cid=AG-PT-122&tabId=AG-PR-1047) is a company-owned application that is specific for gene expression probe design. Selection of probes is based on scores obtained by running different programmes, UNAFold (Markham and Zuker, 2008) for thermodynamic and melting temperature calculations based on sequence composition and BLAST (Altschul et al., 1990) to check the identity and an internally written code. Final scores (called BC scores by eArray) are deduced using a set of empirically heuristics to combine all computed scores (J. Noble, Agilent, personal communication, February 2016).

3. 1. 2. General design parameters

Other calculations are required for probe design, including melting temperature using the nearest neighbour model and the presence of a stable secondary structure using the MFold software (Zuker, 2003). Most software

predicting melting temperature (Tm) and free energy (ΔG) are based on the nearest neighbour model which assumes that the stability of any base pair depends on the orientation and identity of neighbouring base pair (SantaLucia, 1998). A pre-calculated table with nearest neighbour coefficients for the 10 possible Watson-Crick base pairs is used to estimate the free energy of a strand or a duplex by summing up the individual free energies of these possible base pairs (Breslauer et al., 1986). Free energy of mismatching pairs were likewise established and used to estimate the overall free energy in a system (Peyret et al., 1999).

Self-folding of a single-stranded monomer involves changes in energy that is known as free energy or Gibbs free energy (ΔG in Kcal/mol). It is defined as the difference between the energy of the environment or enthalpy (ΔH in Kcal/mol) and the energy of the system to form itself or entropy (ΔS in cal/K per mol). The ΔG of any single strand in the case of self-folding or duplex in the case of hybridisation can be calculated using the standard relationship:

$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, where T is the temperature in Kelvin.

Melting temperature (Tm) corresponds to the temperature at which 50% of the strand population is in a duplex state and 50% is in a single state. The theoretical calculation of Tm relies on state transitions. It is predicted based on the nearest neighbour model using the equation given by Borer et al. (1974):

$Tm = \Delta H / (\Delta S + R \ln CT)$, where R is the gas constant (1.987 cal/K/mol) and CT is the strand concentration (ΔH and ΔS are enthalpy and entropy, respectively).

This allows us to address immediate questions about for example the behaviour profile of hybridisation of designed probes with their respective targets. As such, a bioinformatics tool, ChipCheck (http://chip.chemie.uni-stuttgart.de/software.html), was utilised to predict hybridisation profiles of reference species with probes designed in this study.

3. 2. Materials and Methods

3. 2. 1. Enteropathogen selection

The aetiology of enteritis in cattle and pigs can be complex and may involve different types of microorganisms such as viruses, bacteria and protozoa (Alfieri et al., 2004). The literature was searched (see Literature Review, Chapter 1) and a number of enteropathogens responsible for intestinal infection was selected based on the most commonly cited pathogens causing intestinal infection with an impact on the health of the animal, essentially the most frequently identified major pathogens. Additionally other pathogens, for which no clear evidence has been demonstrated regarding their role in enteritis, were included. However, these would have been isolated alongside ther major pathogens in a high proportion when the animals were diarrhoeic.

3. 2. 2. Database construction

Sequence of genes were searched from the NCBI website (http://www.ncbi.nlm.nih.gov/nucleotide) to construct a full database comprising all useful details for each sequence (Enteropathogen species name and genus, region of the sequence in the genome, length of the gene sequence,

accession number, location in the genome and the coding sequence. A file comprising sequences of interest was created; gene or gene sequences of interest were downloaded in FASTA format and stored electronically. To ensure an extensive coverage of the pathogen genomes, a maximum number of genes/sequence of genes were selected and retrieved from NCBI as shown in Table 3.1., aiming to increase chances of detection in the faecal sample. Some of the sequences in the database include junctions between genes or combination of 2 to 5 genes together. The reason for the choice of the genes in Table 3.1. is that they are the most studied genetic markers in pathogen detection in general, but also the availability of the sequence genes in the public database was an important basis for the selection of such genes. Bovine and porcine host sequences were also introduced in the database, represented by the mitochondrial cytochrome b for each species, allowing identification of infected host species.

Table 3.1. Targeted sequence genes

Viruses	Bacteria	Parasites
Detection genes • Structural • Non structural	• 16S rRNA • 23S rRNA • 16S rRNA-23S rRNA intergenic spacer • Virulence factors • Antibiotic resistance	• 18S rRNA • 5.8S rRNA • Internal transcribed spacer • Virulence factors • Drug resistance

3. 2. 3. Primer design

It is well known that careful primer design can aid PCR optimisation and improve the assay performance significantly (Butler et al., 2001). This step followed probe design since primers were designed using probes as targets to ensure amplification of specific gene sequences, which are complementary to the spotted probes on the array.

Primers were designed using the freely available software on the NCBI, Primer-Blast (www.http://.ncbi.nlm.nih.gov/tools/primer-blast/). To minimise the number of primer pairs to be used, a strategy based on designing pairs of primers that flank a region of gene(s) where multiple probes are present was applied.

3. 2. 4. Probe design

Probes were designed using free online and downloadable software. Unique Probe Selector (UPS 2.0) (www.http://array.iis.sinica.edu.tw/ups/index.php) and eArray (www. https://earray.chem.agilent.com/earray/) are two online available applications, two other applications, Picky (Chou, 2010; Chou et al., 2004) downloaded upon request from the authors and GoArray (Rimour et al., 2005) that was hosted in the author's website, were installed in a computer following authors' instructions.

eArray is a design tool hosted by the Agilent website. Sequence accession numbers were entered and probes were computed and returned to be downloaded. The length of the probes was set to 60 nucleotides. The other parameters used for the probe design are compiled in Appendix V.

Probes were named according to the following format: Software_initial_organism abbreviation_gene_name_number, for example bovine coronavirus membrane probe designed by Picky software was called P_Bov_cor_M_01; viral probes on the ArrayTube were identified only by the organism abbreviation followed by a number (Bov cor_1 for Bovine coronavirus, probe 1).

3. 2. 5. Probe similarities

Because the probe design was performed with 4 software using the same set of sequences, it has been hypothesised that the probe selection was different. This similarity test aimed to visually examine whether the software selected the probes from the same region in the sequence. Randomly selected probes from each software-designed probe batches were aligned against their respective gene sequences in a word document. Probes were attributed colours to highlight their locations in the sequence. Genes used in this test were *Escherichia coli* fimbrial genes (F4 and F5), *Clostridium difficile* toxin A (*tcdA*) gene and Porcine rotavirus VP7. GenBank accession numbers were respectively AY437806.1, S70131.1 and JX498968.1.

3. 2. 6. Thermodynamics of viral probes

In order to assess the stability of the probes designed, the ability of folding and secondary structure formation of the probes (hairpin, bulge, internal loop, external loop and multi-loop) (Fig.3.1) were computed using the DinaMelt web server (Quikfold) (www.http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold)

(Zuker, 2003; Markham and Zuker, 2005) that employs the nearest-neighbour model to calculate the minimum free energy folding, melting temperatures and all possible secondary structures for each probe.

Free energies were also computed for the hybridisation prediction of two sequences using two-state melting application (http://unafold.rna.albany.edu/?q=DINAMelt/Two-state-melting). Probe sequences were entered along with the corresponding target sequence derived from the reference strain of the corresponding pathogen. The target length was set between 491 and 1000 bp. The temperature was set to 50°C which follows the hybridisation temperature of the protocol in this study. The Na^{+} and Mg^{2+} concentrations were set by default to 1M and 0M, respectively. Only viral probes on the Alere platform were subjected to thermodynamic calculations. Output files containing the different conformations of sequences and hybridisation profiles in addition to free energies were downloaded and stored for data analysis.

In order to understand the hybridisation process between viral probes and targets, mismatches were counted and their location determined by aligning the segments, thus the number of hybridised bases was deducted. Mismatches were categorised as 5' terminal, central or 3' terminal as shown in the figure 3.2.

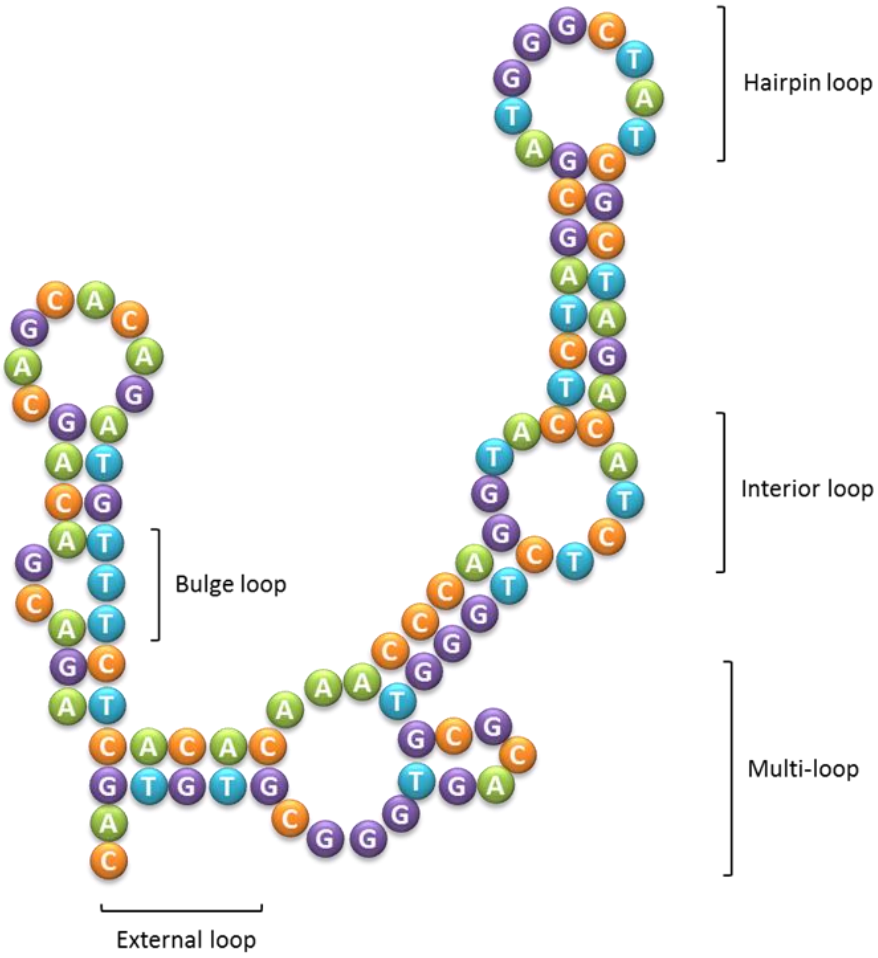
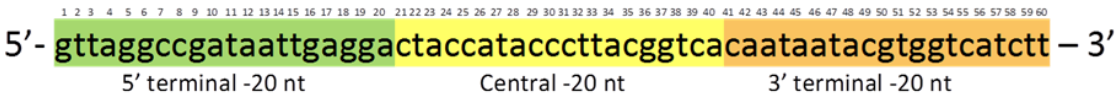


Figure 3.1. Probe secondary structures

Hairpin, interior loop, bulge loop and multi-loop are structures that can occur in single stranded DNA.



60 nt

1-20 : 5' terminal
21-40: Central
41-60: 3' terminal

Figure 3.2. Probe regions identified for base mismatches

3. 2. 7. Hybridisation prediction with ChipCheck

All hybridisation simulations are based on the same principles for many packages such as Visual-OMP (Oligonucleotide Modeling Platform) and Vector NTI that use the nearest neighbour model to calculate the melting temperature and free energy. In addition to hybridisation predictions, these two packages have been used extensively for sequence alignment, sequence analysis, database search and primer design. These two products are powerful and useful for *in silico* simulations as they offer a series of tools for the manipulation of biological molecules, they are also user friendly. No comparison has been made of the various softwares avaiabe, including these two above and ChipCheckII (www.http://http://chip.chemie.uni-stuttgart.de/software.html) (Siegmund et al., 2003), but the latter was selected as the only freely available online tool that predicts strength/extent of hybridisation for DNA chips and was designed only for microarray hybridisation simulation. It also has a graphical tool for supporting the *in silico* array hybridisation. The software estimates hybridisation of probes with targets in solution predicting matched duplexes and detecting expected cross-hybridisation. Files comprising sequences of probes on the viral array with respective molar concentrations and target sequences with respective molar concentrations were prepared. The volume and temperature of the hybridisation solution was set to 100 µl and 50°C, respectively, according to the protocol used in this study. The output result consists of an image of a matrix in a form of an array that shows probes in rows and targets in columns with, at the intersections, numbers corresponding to

the percentages of probes that bind to the targets. The simulation was performed for five viruses, Porcine Epidemic Enteritis Diarrhoea virus (PEDV), Transmissible Gastroenteritis Virus (TGEV), Porcine circovirus (PCV), Porcine parvovirus and Bovine viral diarrhoea virus (BVDV).

3. 2. 8. Statistical analyses

The number and frequencies of sequences employed in the probe design and probes generated by software were determined with Excel 2010. All data sets were tested for their normality, to justify the choice of parametric or non-parametric statistical tests. The numbers of probes designed by the four software (UPS, Picky, GoArray and eArray) for each group of organisms were compared using two way ANOVA using SPSS Statistics 22.0.

Free energies (individual and hybridised probes), folding and mismatches data were compared using non-parametric tests as both distributions were not normal.

Correlations were performed using the Pearson correlation coefficient to assess the performance of Chipcheck in predicting hybridisation profile, compared to free energy hybridisation calculations and signal intensities of corresponding viruses hybridised to the array tube.

Correlations and linear regressions were also carried out using SPSS 22.0. The significance threshold value was set at $P<0.05$ for all performed tests.

The thermodynamic values of the secondary structures for each probe were analysed for their most probable structure to occur using Boltzmann probability equation:

$$P(q) = \frac{e^{-\frac{E(q)}{k_B T}}}{Z}$$

Where E(q) is the energy of state, q (in kt), kB is the Boltzmann constant, T the temperature in Kelvin and Z the partition function.

Graphs and histograms were plotted using Graph Pad Prism 6.05.

3. 3. Results

3. 3. 1. Enteropathogen selection

Fifty four different species and serotypes of pathogens belonging to 29 genera and 17 families are known to cause intestinal disorders in cattle and pigs (Table 3.2).

Table 3.2. Bovine and porcine enteropathogens

Family name	Genus name	Organism name
Coronaviridae	*Betacoronavirus*	Bovine coronavirus
	Alphacoronavirus	Porcine epidemic diarrhea virus (PEDV) Transmissible gastroenteritis virus (TGEV)
	Torovirus	Bovine torovirus Porcine torovirus
Circoviridae	*Circovirus*	Porcine circovirus (PCV1) Porcine circovirus (PCV2)
Picornaviridae	*Kobuvirus*	Bovine kobuvirus
		Porcine kobuvirus
	Enterovirus	Bovine enterovirus
		Porcine enterovirus
	Sapelovirus	Sapelovirus
	Teschovirus	Teschovirus
Caliciviridae	*Norovirus*	Bovine norovirus

		Porcine norovirus
	Nebovirus	Nebovirus
	Sapovirus	Sapovirus
Reoviridae	*Rotavirus*	Bovine rotavirus A Bovine rotavirus B Bovine rotavirus C Porcine rotavirus A Porcine rotavirus B Porcine rotavirus C

Continued

Family name	**Genus name**	**Organism name**
Reoviridae	*Orthoreovirus*	Porcine orthoreovirus
Parvoviridae	*Bocavirus*	Bovine parvovirus Porcine bocavirus
	Parvovirus	Porcine parvovirus
Flaviviridae	*Pestivirus*	Bovine viral diarrhoea virus (BVDV-1) Bovine viral diarrhoea virus (BVDV-2)
Bacteroidaceae	*Bacteroides*	*Bacteroides fragilis*
Campylobacteraceae	*Campylobacter*	*Campylobacter jejuni* *Campylobacter coli*
Clostridiaceae	*Clostridium*	*Clostridium difficile* *Clostridium perfringens*
Enterobacteriaceae	*Escherichia*	*Escherichia coli* EPEC *Escherichia coli* ETEC *Escherichia coli* STEC *Escherichia coli* EAEC
	Salmonella enterica	*Salmonella* Typhimurium *Salmonella* Enteritidis Salmonella Dublin Salmonella Derby Salmonella Newport *Salmonella* Typhisuis *Salmonella* Cholerasuis

	Yersinia	*Yersinia enterocolitica*
Brachyspiraceae	*Brachyspira*	*Brachyspira hyodysenteriae* *Brachyspira pilosicoli*
Desulfovibrionaceae	*Lawsonia*	*Lawsonia intracellularis*
Trichuridae	*Trichuris*	*Trichuris suis*
Cryptosporidiidae	*Cryptosporidium*	*Cryptosporidium* spp.
Giardiidae	*Giardia*	*Giardia* spp
Eimeriidae	*Eimeria*	*Eimeria* spp.
	Isospora	*Isospora suis*

Figure 3.3 shows the proportions of each group of pathogens selected. Viruses represented the highest proportion with more than half of the total number of enteropathogens (53.70%). Bacteria and parasites represented 37.04% and 9.26%, respectively.

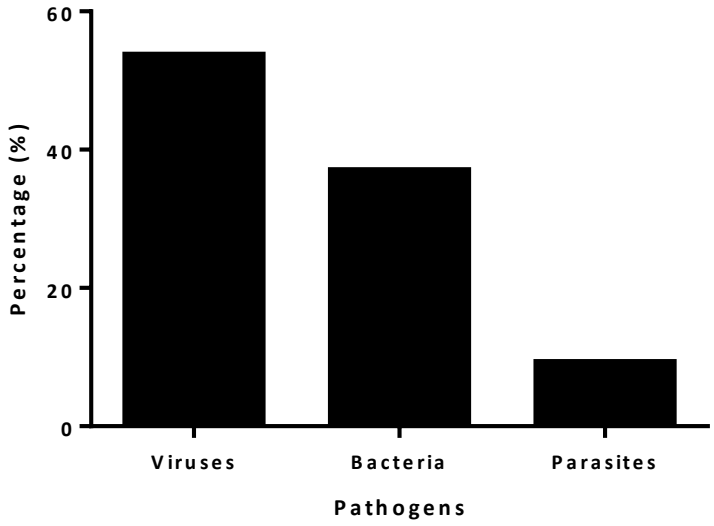


Figure 3.3. Proportions of enteropathogens targeted

3. 3. 2. Database construction

The database constructed comprised 2205 sequences from complete or partial gene sequences representing 54 species or serotypes of enteropathogens. The

number of sequences in the database was reduced to 1921 sequences with 284 gene sequences excluded from the probe design because of redundancy.

Gene sequences from bacterial species were 49.09% of the total number of sequences representing the highest proportion, followed by viruses with 44.61% (Fig. 3.4). The part of sequences occupied by parasites and host genes in the database was 5.10% and 1.20%., respectively.

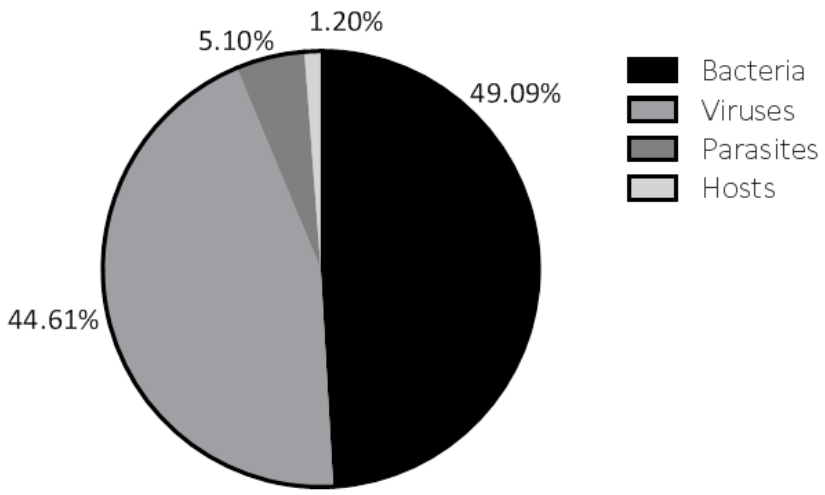


Figure 3.4. Distribution of the finally selected sequences from genes of the different enteropathogens in the database

3. 3. 3. Primer design

A set of primer pairs was designed for each pathogen based on the location of the probe in the genome. A total of 100 pairs of primers were designed with a final product length of 400-1928 bp targeting different genes (Appendix IV). The number of probes flanked by primers varied from 1 to 47. Figure 3.5 shows a pair of primers framing nine probes of PEDV spike, envelope and membrane genes.

Porcine epidemic diarrhea virus strain CV777, complete genome

GenBank: AF353511.1

GenBank Graphics

>gb|AF353511.1|:24568-26494 Porcine epidemic diarrhea virus strain CV777, complete genome

CTTGAGTGGCTCAACCGAGTTGAGACATACATCAAGTGGCCGTGGTGGGTTTGGTTGATCATTGTTATTG
TTCTCATCTTTGTTGTGTCATTACTAGTGTTCTGCTGCATTTCCACGGGTTGTTGTGGATGCTGCGGTTG
CTGCGGTGCTTGTTTTTCAGGTTGTTGTAGGGGTCCTAGACTTCAACCTTACGAAGCTTTTGAAAAGGTC
CACGTGCAGTGATGTTTCTTGGACTTTTTCAATACACGATTGACACAGTTGTCAAAGATGTCTCGAAGTC
TGTCAACTTGTCTTTGGATGCTGTCCAAGAGTTGGAGCTCAATGTAGTTCCAATTAGACAAGCTTCAAAT
GTGACGGGTTTTCTTTTCACCAGTGTTTTTGTTTACTTCTTTGCACTGTTTAAAGCGTCTTCTTTGAGGC
GCAATTATATTATGTTGGCAGCGCGTTTTGCTGTCGTCTTTCTCTATTGCCCACTTTTATATTACTGTGG
TGCACTTTTAGATGCAACTATTATTTGTTGCGCACTTATTGGCAGGCTTTGTTTAGTCTGCTTTTACTCC
TGGCGCTATAAAAATGCGCTTTTTATTATCTTTAATACTACGACACTTTCTTTTCTCAATGGTAAAGCAG
CTTATTATGACGGCAAATCCATTGTGATTCTAGAAGGTGGCGACCATTACATCACTTTTGGCAACTCTTT
TGTTGCTTTCGTTAGTAACATTGACTTGTATCTAGCTATACGTGGGCGGCAAGAAGCTGACCTACATCTG
TTGCGAACTGTTGAGCTTCTTGATGGCAAGAAGCTTTATGTCTTTTCGCAACATCAAATTGTTGGCATTA
CTAATGCTGCATTTGACTCAATTCAACTAGACGAGTATGCTACAATTAGTGAATGATAATGGTCTAGTAG
TTAATGTTATACTTTGGCTTTTCGTACTCTTTTTCCTGCTTATTATAAGCATTACCTTCGTCCAATTGGT
TAATCTGTGCTTCACTTGTCACCGGTTGTGTAATAGCGCAGTTTATACACCTATAGGGCGCCTGTATAGA
GTTTATAAGTCTTACATGCGAATTGACCCCCTCCCCAGTACTGTTATTGACGTATAAACGAAATATGTCT
AACGGTTCTATTCCCGTTGATGAGGTGATTGAACACCTTAGAAACTGGAATTTCACATGGAATATCATAC
TGACGATACTACTTGTAGTGCTTCAGTATGGCCATTACAAGTACTCTGTGTTCTTGTATGGTGTCAAGAT
GGCTATTCTATGGATACTTTGGCCTCTTGTGTTGGCACTGTCACTTTTTGACGCATGGGCTAGCTTCCAG
GTCAACTGGGTCTTTTTCGCTTTCAGCATCCTTATGGCTTGCATCACTCTTATGCTGTGGATAATGTATT
TTGTCAATAGCATTCGGTTGTGGCGCAGGACACATTCTTGGTGGTCTTTCAATCCTGAAACTGACGCGCT
TCTCACTACTTCTGTGATGGGCCGACAGGTCTGCATTCCAGTGCTTGGAGCACCAACTGGTGTAACGCTA
ACACTCCTTAGTGGTACATTGCTTGTAGAGGGCTATAAGGTTGCTACTGGCGTACAGGTAAGTCAATTAC
CTAATTTCGTCACAGTCGCCAAGGCCACTACAACAATTGTCTACGGACGTGTTGGTCGTTCAGTCAATGC
TTCATCTGGCACTGGTTGGGCTTTCTATGTCCGGTCAAAACACGGCGACTATTCAGCTGTGAGTAATCCG
AGTGCGGTTCTCACAGATAGTGAGAAAGTGCTTCATTTAGTCTAAACAGAAACTTTATGGCTTCTGTCAG
CTTTCAGGATCGTGGCCGCAAACGGGTGCCATTATCTCTCTATGCCCCTCTTAGGGTTACTAATGACAAG
CCCCTTTCTAAGGTACTTGCAAACAACGCTGTACCCA

PRIMER U_PEDV_M_08 U_PEDV_M_09 U_PEDV_E_03
U_PEDV_E_01 U_PEDV_E_02 U_PEDV_M_10 U_PEDV_M_06
U_PEDV_E_M_02 U_PEDV_M_05

Figure 3.5. Primers and probes in PEDV sequence

Forward and reverse primers are highlighted with yellow and the probes (60 nucleotides) are highlighted with corresponding colours at the bottom of the sequence – Probes U_PEDV_E_02 and U_PEDV_M_10 and probes PEDV_E_M02 and PEDV_M_05 are overlapping.

3. 3. 4. Probe Design and microarray fabrication

Based on the gene sequences in the database, 15993 probes were designed using the four different algorithms (UPS, Picky, GoArray and eArray) that use different strategies to choose and assess the probes from a submitted set of sequences. The four tools were applied on the same target sequences.

Data from the software used and probes designed for the enteropathogens are illustrated in Fig. 3.6 and Fig 3.7. There is a clear trend in a decreasing number of probes designed by UPS, followed by eArray, Picky and GoArray. As illustrated in figure 3.6, the online tools UPS and eArray allowed the design of respectively 37.41% and 29.90% probes. Lower percentages were noticed with Picky (21.13%) and GoArray (11.76%).

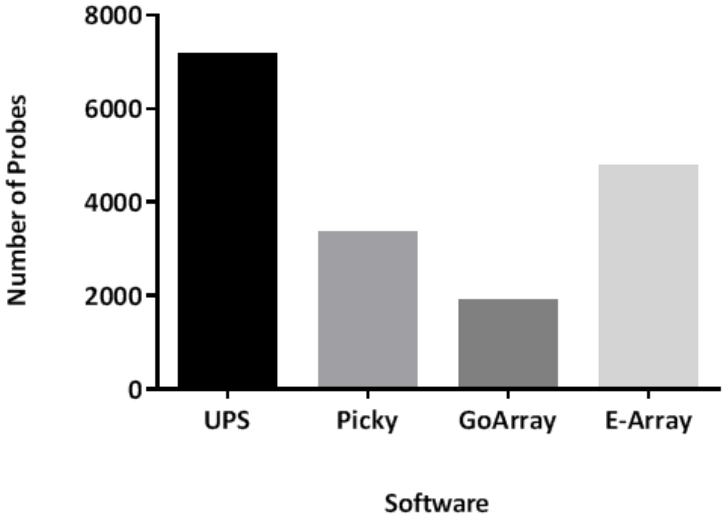


Figure 3.6. Total number of designed probes by software

The details of the four softwares are illustrated in figure 3.7, where the overall proportion of probes showed almost the same trend as the number of designed

probes by software. Amongst the 15993 designed probes, the proportions of bacterial probes were the largest with the highest number achieved by UPS (3125 probes, 19.54%) followed by eArray (2016, 12.61%), Picky (1760, 11%) and GoArray (935, 5.85%). An equally important variation of viral probes by software was noted, showing 15.28% (2444 probes) for UPS, 17.93% (2388 probes) for eArray, 9.47% for Picky (1514 probes), and 5.17% (827 probes) for GoArray. Probes designed from parasitic and host gene sequences had the lowest frequencies varying from 0.01% to 2.38%. The final number of probes in all groups of organisms were different showing high significance ($P<0.001$) although no significant difference was noted between software ($P=0.059$). The number of generated probes per sequence per software was set up to five for UPS and Picky. UPS conforms to the user's specifications. However, Picky seemed to produce a variable number of probes per sequence but this was always fewer than five. eArray and GoArray designed three and one probe for each sequence respectively.

The number of probes designed for each pathogen per software for Agilent and Alere platforms are represented in Appendixes VI and VII.

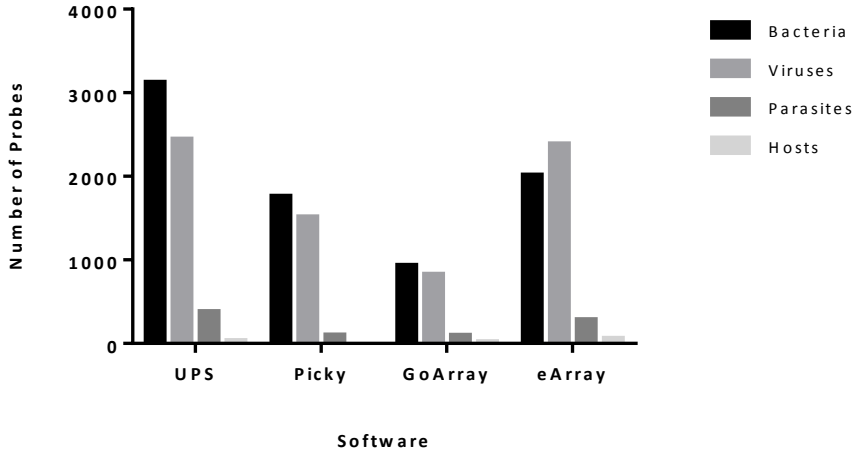


Figure 3.7. Distribution of final probes designed by software and according to pathogen type

The 15993 probes designed by the four software were synthesised in-situ in duplicate on slides comprising 4x44k of 60-mer features (Agilent). The slides contain four hybridisation wells that allow four hybridisation reactions. The probes were randomly-printed on the slide, as randomisation is a feature layout option available in the microarray design order. Agilent introduced 1417 controls in each microarray, which consisted of three types of probes:

- BrightCorner probes, used for orientation purposes. These probes are placed in the corners of the array with a different pattern for each corner.
- DarkCorner probes, used for orientation purposes in the array corners. These, along with the bright corner probes, make up the corner-specific patterns.

- Negative controls, usually highly replicated on the array and used to measure element background. These probes form a hairpin and do not hybridize well with labeled samples of any species.

Selected probes of viral pathogens that were designed with UPS (201 probes with three biotin markers, which are biotinylated non-specific oligonucleotide probes) were each printed at 15µM onto an ArrayTube (AT) platform by Alere™ Technologies. The probes were synthesised commercially with NH2 modification at the 3' end, no modification at the 5' end, purification with HPLC, 0.04 mmol scale, and absolutely biotin-free (Metabion International, Jena, Germany). The probes were spotted in an orderly manner according to the list of probes provided and sent to the company. Randomisation is not necessary for such a small surface area.

3. 3. 5. Probe similarities

In order to present an example of the method of probe selection by software, the location of each probe has been identified in the same gene sequence. Results were illustrated in figures 3.8, 3.9, 3.10 and 3.11.

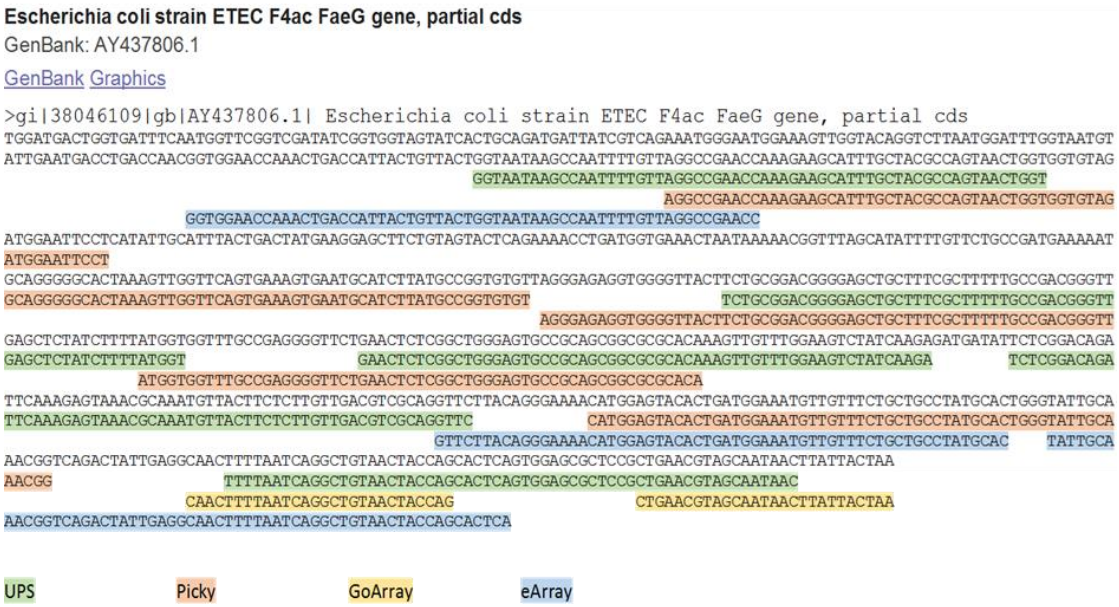


Figure 3.8. Probe locations in F4 fimbrial subunit gene sequence (*faeG*) of *Escherichia coli.*

Probes designed by the four software are labelled with colours UPS: Green, Picky: Pink, GoArray: Yellow and eArray: Blue.

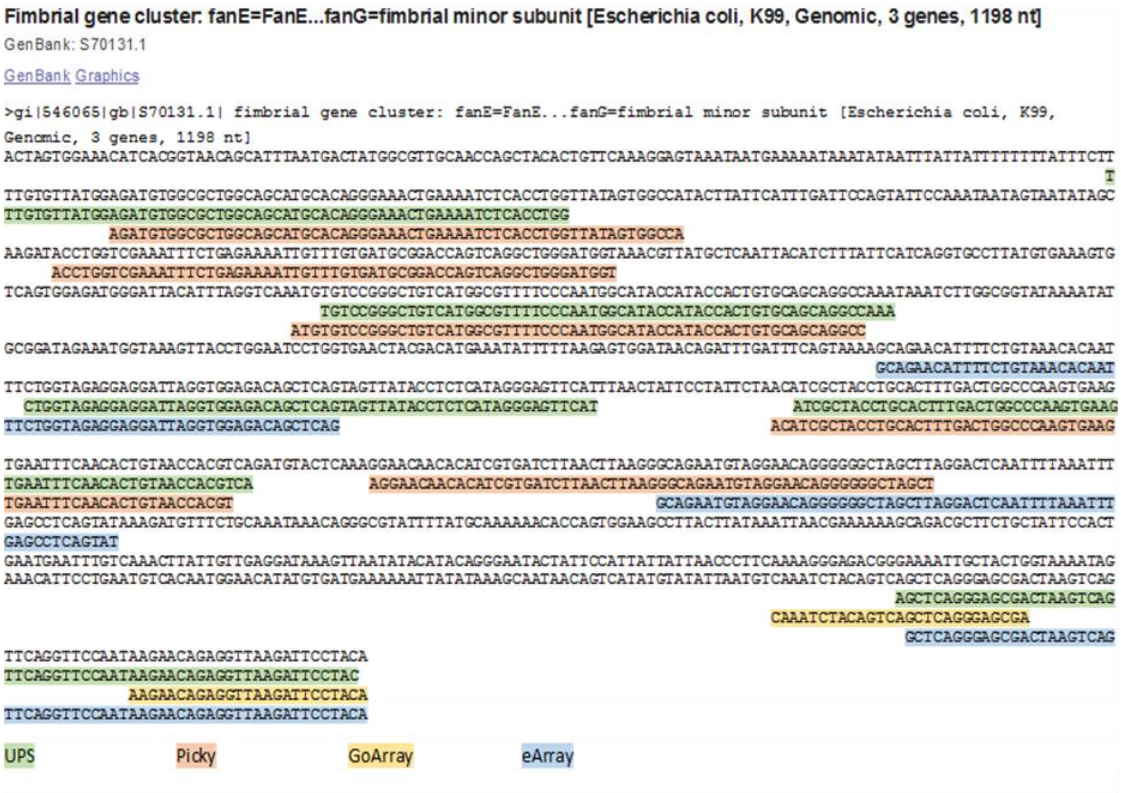


Figure 3.9. Probe locations in fimbrial subunit gene F5 sequence (*fanG*) of *Escherichia coli*.
Probes designed by the four software are labelled with colours UPS: Green, Picky: Pink, GoArray: Yellow and eArray: Blue.

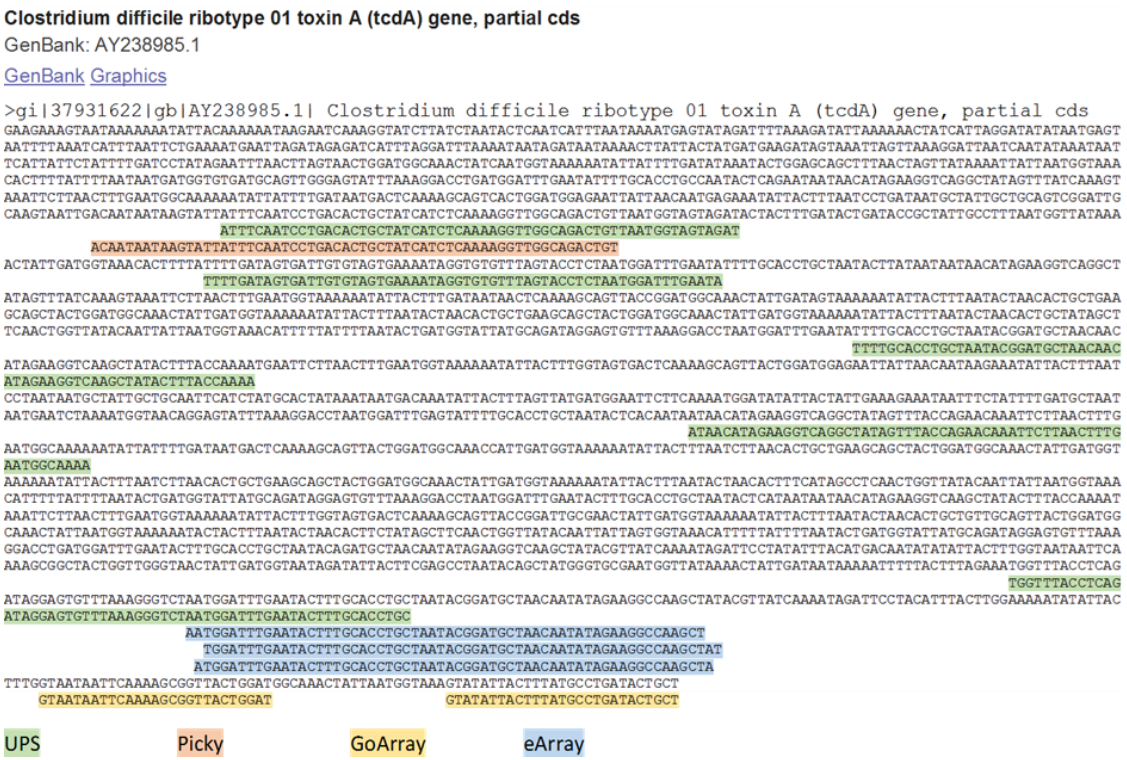


Figure 3.10. Probe locations in toxin A gene sequence (*tcdA*) of *Clostridium difficile*.

Probes designed by the four software are labelled with colours UPS: Green, Picky: Pink, GoArray: Yellow and eArray: Blue.

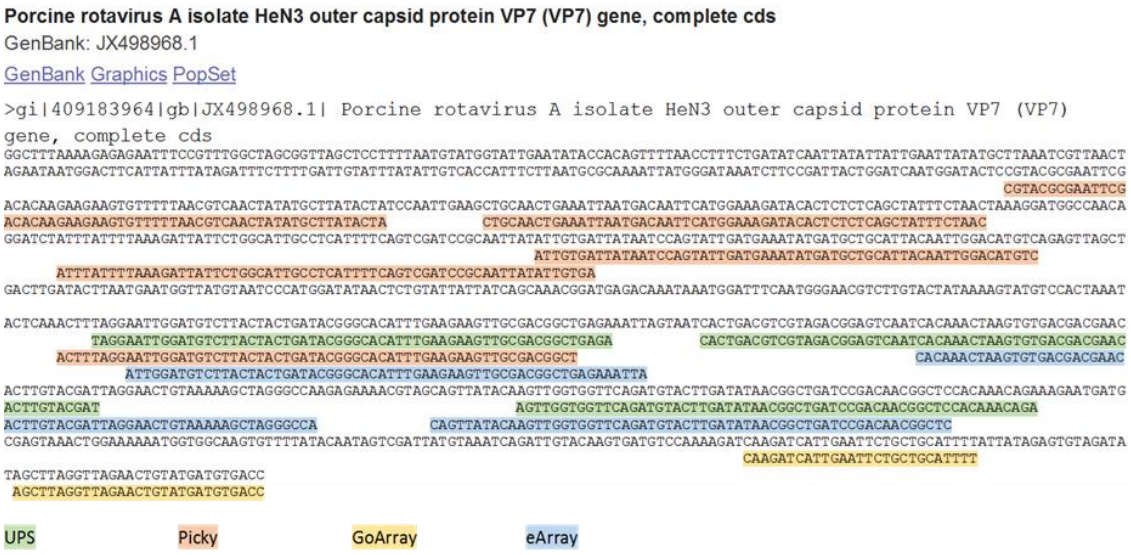


Figure 3.11. Probes location in VP7 gene sequence of Porcine rotavirus A. Probes designed by the four software are labelled with colours UPS: Green, Picky: Pink, GoArray: Yellow and eArray: Blue.

The use of four different softwares with their corresponding distinct approaches enabled the coverage of the entire sequence as illustrated in figures 3.8, 3.9, 3.10 and 3.11. Probes from UPS and Picky frequently overlapped with eArray probes which also overlapped with UPS and Picky probes on occasions.

One of the observations made on probe choice by UPS is that the selection extended throughout the whole sequence. However, for eArray and more specifically GoArray, the 3' region seemed to be the predilection site.

Few to hundreds of bases sometimes separated probe sequences, mainly for UPS; conversely Picky and eArray selected their probes to be only 1 to 4 bases apart (Fig. 3.8).

Finally, it appeared that eArray targets the same region for the whole set of probes with only one base difference, as illustrated in the *Clostridium difficile tcdtA* gene sequence (Fig. 3.10).

3. 3. 6. Thermodynamics of viral probes

3. 3. 6. 1. Folding and Secondary structures

The free energies, melting temperatures and secondary structures were determined for ArrayTube probes (Alere platform) only using the MFold online tool.

Secondary structures formed by probes are a major issue for duplex formation. Figure 3.12 shows the number of possible foldings of probes. The 60-mer oligonucleotides were prone to 1 to 12 secondary structures as indicated by the results in this study.

Among the total number of possible secondary structures, the folding average of Porcine norovirus, Bovine torovirus, PCV, TGEV and Porcine rotavirus showed the highest number of foldings with an average of more than 4 foldings per probe. Two PCV probes (Por_cir2_3 and Por_cir1_2) exhibited respectively 12 and 10 possible conformations each at the hybridisation temperature (50°C). A low number of probes (23, 11.44%) had a unique predicted structure. For the 201 probes, it appeared that among a total of 743 configurational states, 1612 predicted secondary structures were possible. The mean numbers of the 4 distinct secondary structures were statistically different from each other ($P<0.001$) (Fig. 3.13) using one way ANOVA. Also all viral probes had at least one folding structure, with all having at least 1 hairpin. More than half of

the probes (57.71%) showed at least one interior loop, 30.35% had at least one bulge structure and 1.99% a multi-loop structure. Hairpin loops are the most frequent structure that is likely to form during the probe folding (Fig.3.13) with 18.90% of probes presenting a maximum of 3 different hairpin structures at a time, 4.47% of probes showed a maximum of 2 bulges and 2.98% of probes were predicted to have 3 interior loops in one configuration.

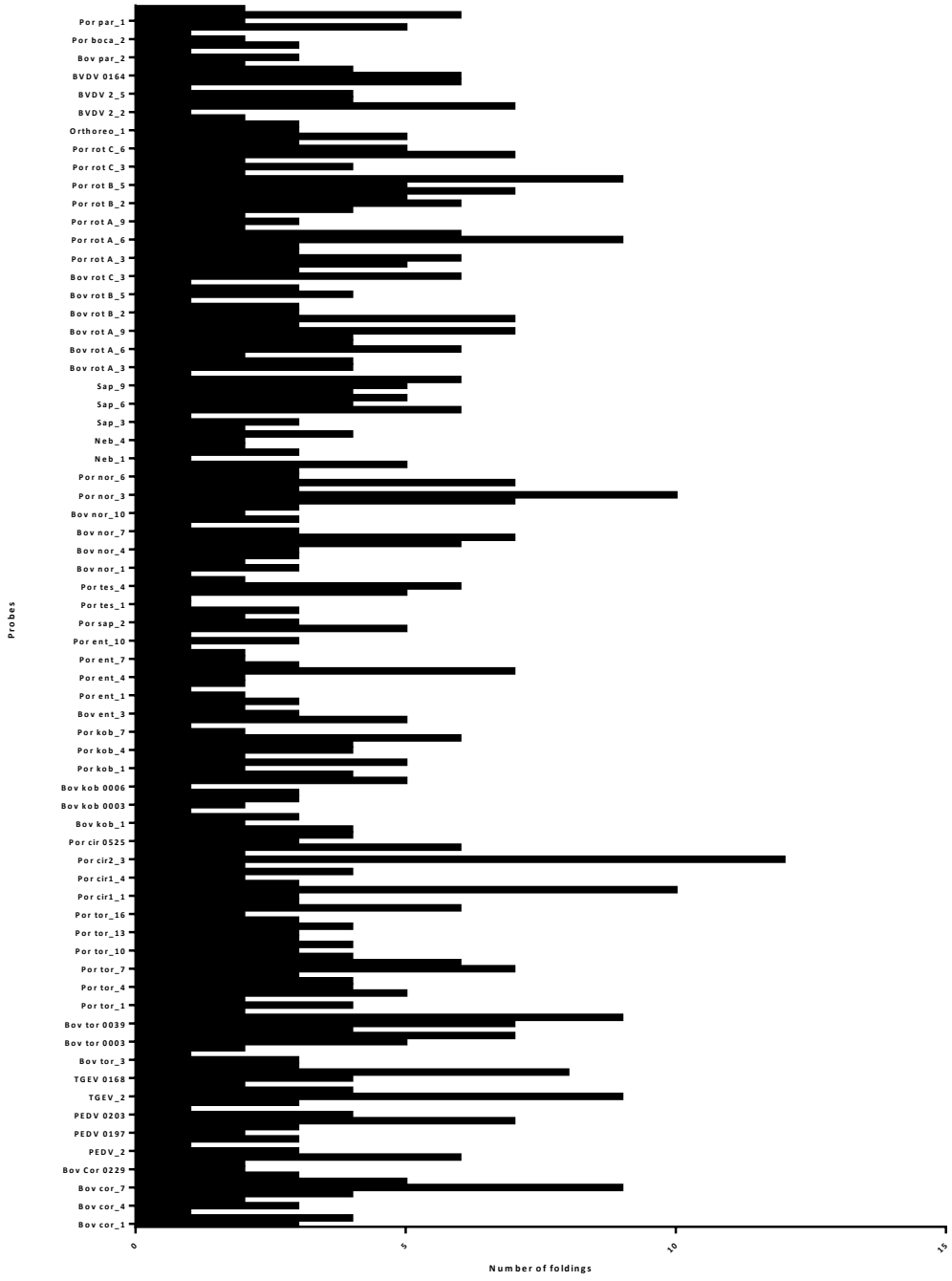


Figure 3.12. Number of individual probe folding

Probes show more than one folding structure, the number of conformation varied from 1 to 12 secondary structures that can be interior loop, bulge, hairpin loop or multi-loop.

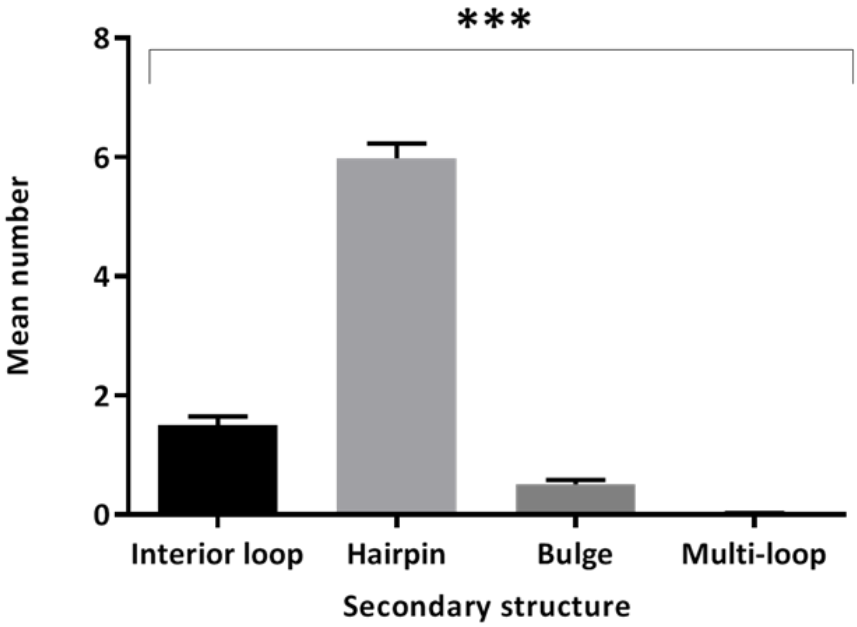


Figure 3.13. Mean number of secondary structures with SEM.
The most frequent secondary structure was the hairpin loop. All the probes had at least 1 hairpin loop. The mean number of the diverse structures was significantly different (P<0.001).

3. 3. 6. 2. Free energy of virus probes

The Boltzmann probability equation was used to determine the most probable among the predicted free energies (Appendix VIII). Positive free energies were removed from the calculation as they imply a constant unstable state. A total of 13 (6.46%) probes were expected to have only positive energies with different configurations.

Figure 3.14 illustrates free energies (ΔG) of the probes. Free energy values varied from -8.30 to +1.46 kcal/mol. Free energy data of probes are not normally distributed, hence the use of non-parametric test (Kluskal-Wallis test) which showed that the free energies among the probes were not significantly different (p=0.487). Negative free energies of -1 to -2 kcal/mol were observed

for 24.38% of the total number of probes and 21.39% of probes had a free energy inferior to -4 kcal/mol. Correlation between the mean of each probe free energies and number of foldings using the Spearman correlation test showed a moderate positive relationship between the two parameters ($r=0.580$, $P<0.01$).

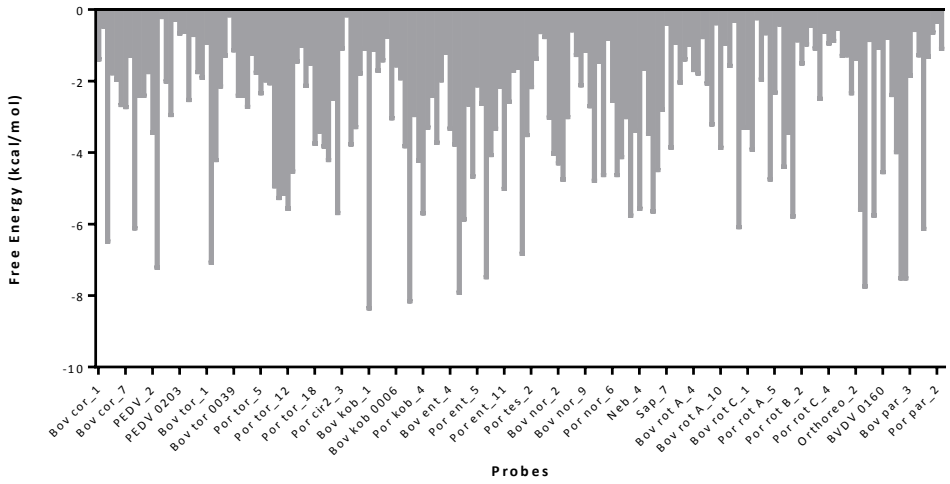


Figure 3.14. Free energies of virus probes.
The most frequent free energies displayed by the probes were comprised between -1 and -2 kcal/mol.

3. 3. 6. 3. Hybridisation prediction of virus probes with targets

a. Hybridisation prediction using free energies

Hybridisation prediction of the virus probes with the reference genome target of the corresponding virus was performed using a two-state melting algorithm. Free energy, mismatches and their location on the probes were computed for each probe. The probe-target free energies with number of mismatches are illustrated in Appendix IX

Free energy data were categorised based on ascendant free energies, and the number of hybridised probes were calculated for each category (Table 3.3).

Table 3.3. Distribution of number and percentage of hybridised probes per range of free energies

Free energy range (kcal/mol)	No	%
> -70	2	0.99
-60 to -70	39	19.40
-50 to -60	53	26.37
-40 to -50	22	10.94
-30 to -40	21	10.45
-20 to -30	25	12.44
-10 to -20	21	10.45
0 to -10	18	8.95

The free energies of probes bound with their corresponding targets varied from -7.1 to -72 kcal/mol. Table 3.3 shows that for each of the four free energy categories, -10 to -20, -20 to -30, -30 to -40 and -40 to -50, almost 10% of the total number of ΔG hybridisations fell in each category. The highest rate of probes (26.37%) was represented by hybridisations with free energies in the interval of -50 to -60 kcal/mol. However, the comparisons of percentages using the Chi-Square test indicated that there was no significant difference between the different groups ($p=0.993$).

- **Correlation of duplex free energy with mismatches**

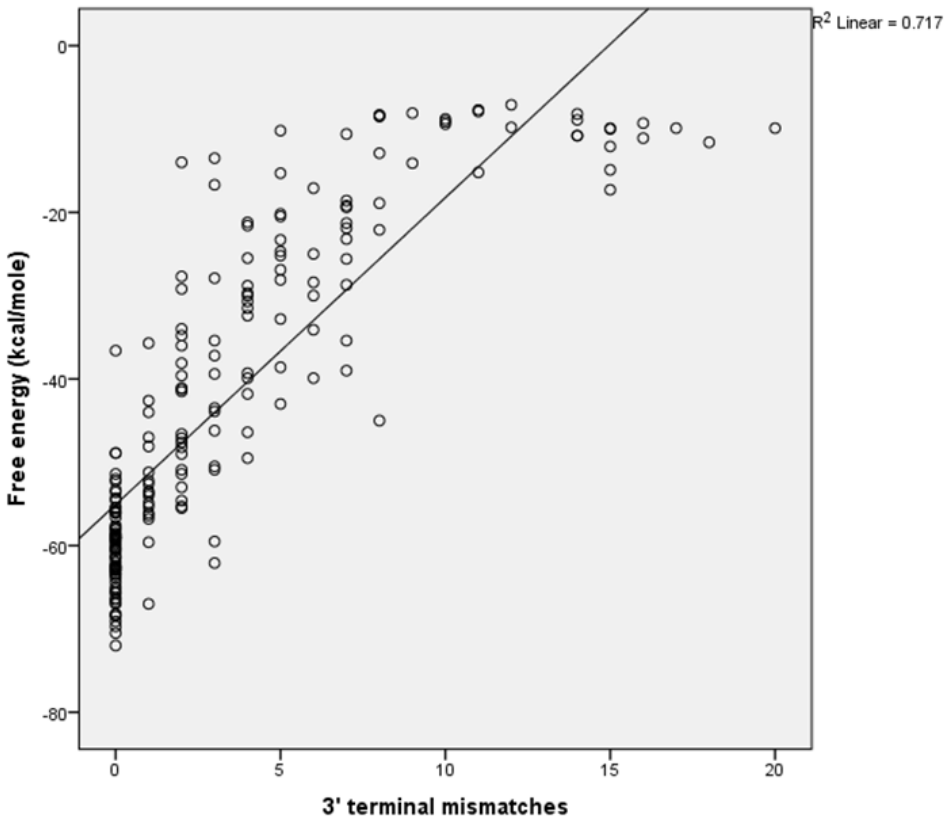
Perfect match probes (PM) were defined as having no mismatches with target sequence while mismatch probes (MM) had at least one mismatch with the target. The frequency of MM probes was higher than PM probes with 71.7% and 28.9%, respectively, and the difference was highly significant ($p<0.001$).

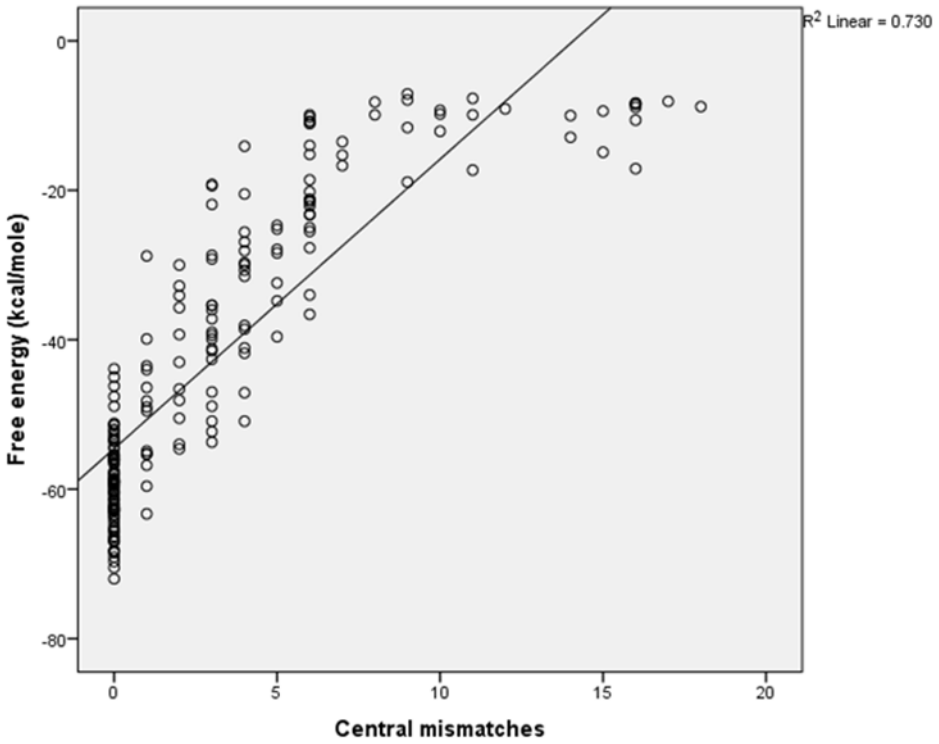
A non-parametric correlation test of free energy with mismatches between probes and targets using the Spearman test showed that free energies of hybridisation and mismatches were positively correlated ($r=0.943$, $p<0.001$). Likewise, the number of bases of the probes bound to the targets were negatively correlated with hybridisation free energies ($r=-0.940$, $p<0.001$)

b. Effect of mismatch positions on the duplex free energy

To better understand the influence of the position of mismatches on the binding energies, linear regression was performed to assess whether mismatches located in the centre or extremities of the probe impacted the free energy values, and consequently the ability of the hybridised duplex to remain stable.

The numbers of mismatches in different locations of the probes and corresponding energies were plotted in figure 3.15. Linear regression analysis showed that there is a clear relationship between mismatches and binding free energies. When one mismatch is situated in the 5' region of the probe, the free energy increases by 3.5 kcal/mol ($p<0.001$), while when it occurs in the 3' region ($p<0.001$), free energy is likely to increase by 3.7 kcal/mol. However, when the mismatch is in the central area it increases by 3.9 kcal/mol ($p<0.001$).


R² Linear = 0.717
Free energy (kcal/mole)
0
-20
-40
-60
-80
0
5
10
15
20
3' terminal mismatches


R² Linear = 0.730
Free energy (kcal/mole)
0
-20
-40
-60
-80
0
5
10
15
20
Central mismatches

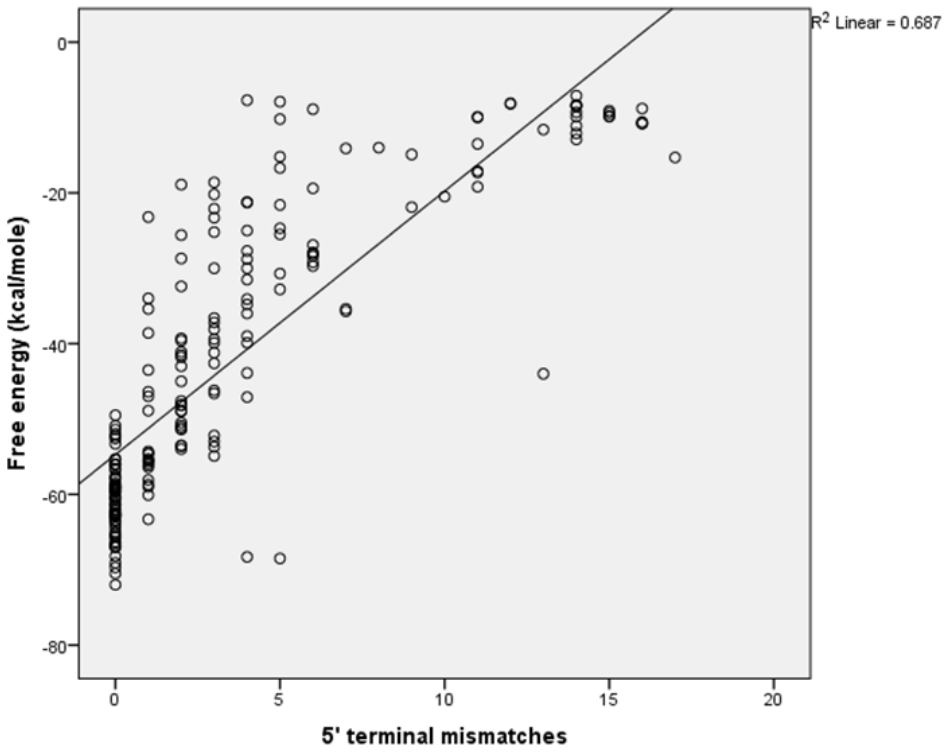


Figure 3.15. The linear relationship between mismatches and free energy
Top panel, 3'mismatches; middle panel, central mismatches; bottom panel, 5' Mismatches.

c. *In silico* hybridisation prediction and comparison with hybridisation free energies and ArrayTube hybridisation signal intensities

Simulation of tube array hybridisation was also performed *in silico* with two sets of probes and target sequences using the ChipCheck II software. The simulation was run for PEDV, TGEV, PCV, PPV and BVDV, five viruses for which hybridisation data from ArrayTubes were available (Chapter 5). Binding free energies were also predicted *in silico* for the probes of the same viruses (Table 3.4).

Table 3.4. Results of free energy, predicted and experimental signal intensities

Probes	Free energy (kcal/mol)	ChipCheck predicted SI	Experimental SI
PEDV_1	-62.6	97.614	0.28
PEDV_2	-63.3	99.977	0.778
PEDV_3	-62.9	93.57	0.624
PEDV_4	-52	54.026	0.788
PEDV 0197	-59.5	95.424	0.696
PEDV 0199	-53.3	51.864	0.334
PEDV 0201	-59	96.501	0.608
PEDV 0203	-57.8	99.355	0.745
PEDV 0205	-62.9	98.742	0.738
TGEV_1	-62.6	99.836	0.739
TGEV_2	-48.9	87.473	0.749
TGEV 0163	-59.1	81.219	0.723
TGEV 0166	-53.6	85.085	0.765
TGEV 0168	-52.6	85.417	0.792
Por cir1_1	-67	20.2661	0.008
Por cir1_2	-61.5	48.0002	0.745
Por cir1_3	-66.8	21.849	0.490
Por cir1_4	-70.5	99.6996	0.733
Por cir2_1	-69.7	87.0703	0.820
Por cir2_2	-44	99.9863	0.818
Por cir2_3	-68.3	50.3627	0.813
Por cir 0521	-57.6	62.2846	0.332
Por cir 0523	-66.5	99.7115	0.552
Por cir 0525	-69.1	44.2566	0.877
Por cir 0527	-66.3	98.153	0.879
Por cir 0529	-58.7	54.4174	0.698
Por par_1	-61.2	98.92	0.727
Por par_2	-58.5	48.27	0.727
Por par_3	-59	48.72	0.783
BVDV 2_1	-25	83.94	0.00007
BVDV 2_2	-34	28.386	0.242
BVDV 2_3	-9.8	99.139	0.001
BVDV 2_4	-67	47.625	0.00151
BVDV 2_5	-25.5	97.115	0.0015
BVDV 0160	-60.4	54.965	0.770
BVDV 0162	-60.5	81.976	0.524
BVDV 0164	-56.6	46.997	0.191
BVDV 0166	-60.7	39.608	0.271

SI: Signal intensity

Correlations between these three sets of data showed that predicted free energy and predicted signal intensity using Chipcheck of PEDV probes were negatively highly correlated (r=-0.875, p<0.01). All parameters were not correlated for the other virus probes (Table 3.5).

Table 3.5. Correlation results

Virus probe	Parameter	r	Significance
PEDV	Free energy / Chipcheck prediction SI	-0.875**	0.002
	Free energy/ Experimental SI	-0.42	0.914
	Chipcheck prediction SI / Experimental SI	0.216	0.577
TGEV	Free energy / Chipcheck prediction SI	-0.491	0.401
	Free energy/ Experimental SI	-0.549	0.338
	Chipcheck prediction SI / Experimental SI	-0.117	0.852
PCV	Free energy / Chipcheck prediction SI	0.196	0.542
	Free energy/ Experimental SI	0.025	0.938
	Chipcheck prediction SI / Experimental SI	0.503	0.096
PPV	Free energy / Chipcheck prediction SI	-0.986	0.106
	Free energy/ Experimental SI	0.342	0.778
	Chipcheck prediction SI / Experimental SI	-0.49	0.672
BVDV	Free energy / Chipcheck prediction SI	0.612	0.080
	Free energy/ Experimental SI	0.551	0.124
	Chipcheck prediction SI / Experimental SI	-0.294	0.445

SI: Signal intensity, **: p<0.01, r: coefficient of correlation

3. 4. Discussion

In total, 15993 probes were designed for the 54 pathogens species/serotypes involving 1921 specific gene sequences. The highest number of probes was achieved by UPS and eArray compared to Picky and GoArray software. There was no significant difference between the number of probes generated by UPS, Picky and GoArray for the same sequences ($p=0.059$). However, the numbers of probes in each group of organisms per software were significantly different ($P<0.001$).

UPS and Picky compute a selection of probes as a starting point using the same model through suffix trees structures. Filters for the best probes using thermodynamic calculations are performed by the mean of the nearest neighbour model by the four softwares. However, Picky executes these calculations only for sequences that have 75% or more identity. For UPS, and GoArray, probes are tested for specificity and cross-hybridisation (BLAST) and for secondary structure formation (MFold) via external software links. Likewise, eArray uses an external link to test probes and an additional written code for their specificity. In Picky, these parameters are computed using a suffix array to uncover similarities. However, the user is required to provide a non-target sequence set as a background. This aspect contributes highly to increasing the running speed of the software in which Picky is distinguished by its execution time. Besides, Picky determines similarities among gene sequences by showing the shared probes and the reverse complement of each sequence is also considered in all computational calculations.

For assessment of specificity, Kane's conditions, stating that the complementarity string to non-target sequences should be less than 75% and the stretches of continuous complementary, non-target sequences should be less than 15 bp to avoid cross-hybridisation (Kane et al., 2000), were applied in the probe choice for three software (UPS, Picky and GoArray). Nevertheless, UPS uses less strict criteria increasing the first and the second Kane's condition to ≤ 85% and ≥ 17 bp, respectively. A recent study demonstrated that a complementary stretch to non-target sequence as short as 12 bp may result in a significant signal, especially in the absence of the target sequence in the hybridisation solution (Garhyan et al., 2013). There is no information about the use of Kane's conditions in eArray probe design.

The software packages were easy-to-use but differed in the numbers of probes computed. UPS, produced five probes as instructed, whereas Picky generated less than five probes despite the number of probes per sequence entered in the software interface was five. GoArray yielded only one probe per sequence and no preferences were allowed by this software. Alongside Picky, this software permitted to make alteration to the GC content, range of Tm, and sequence similarity parameters. All the software offered the possibility to change the probe sequence size and salt concentration. UPS had many options available such as Unique probe within a group, Unique probe in the specific organism, Unique probe based on pangenomic level and Unique probe based on user's defined organism. For the four software, output files consist of downloadable list of probes with sequence analysis parameters such as TM,

GC%, position of the probe on the submitted sequence and the accession number. Other parameters are provided by UPS only, including the extent of cross-hybridisation, with the length of the hit sequence and the E-value and the overlap ratio. UPS and Picky were the two software that categorise the probes into "best probe" and "others" for UPS and "unique" and "shared" for Picky. In terms of speed of processing, Picky was the fastest software, followed by GoArray and UPS, which is an online application that computes probes from sequences submitted from different parts of the world.

Probes uniformly distributed throughout the target permit an important coverage of the sequence, which consequently should improve binding of targets to their corresponding probes and probably increases identification accuracy. However, it represents a negative aspect of primer design when using specific amplification prior to hybridisation onto the array. Based on the sample set of selected probes to assess degrees of similarity between probes, UPS, Picky appeared to choose common regions to select probes. GoArray and eArray targeted the 3' region of the sequence, which is mainly because these algorithms were implemented for gene expression microarray protocols because cDNA fragments authentically represents the 3' end as synthesis starts from polyA tail of mRNA (Dufva et al., 2009). In microbial detection microarrays, and particularly in this study, the methodology is different; targets are fairly long, and binding at the 3' end which is the surface-immobilised end might be challenging as the target needs a reasonable degree of freedom to reach the probe. However, approaches employed by the different software for

selecting probes were important to understand and compare their performance.

Target sequence detection involved the use of primers, which were designed using Primer-BLAST, an automatic online designing tool provided by NCBI that allows finding specific primers for a submitted query sequence.

The high numbers of oligonucleotide probes designed required the design of primers that allows the amplification of a sequence from the genome comprising several target sequences to prevent the use of a high number of primer pairs, eventually multiplexing a minimum number of primer pairs and knowing the limitations of the technique (Lindroos et al., 2002). Accordingly, 100 primer pairs were designed to amplify 400 to 1928 bp amplicons in which 1 to 47 probes were more likely to be present, for instance primers were designed to specifically amplify a 1928 bp fragment of PEDV that is expected to enclose nine viral probes (Fig. 3.5).

Virulence, ribosomal subunits and antibiotic resistance genes were the selected genes for the probe design. Other species-specific regions on the bacterial genome such as species-specific repeats (Koressaar and Remm, 2013) could also be exploited for a more accurate identification.

Thermodynamic parameters (free energy and secondary structure formations) of virus probes were predicted with M Fold (Markham and Zuker, 2005). Overall, free energy values at 50°C varied from -8.30 to +1.46 kcal/mol and were negative for 93.54% of probes and positive for 6.46% of probes. There was no significant difference between free energies among probes.

Oligonucleotides with negative free energy are more stable than with positive energy (Forsdyke, 2007), implying that the majority of folded probes would be unable to hybridise as conformational structures would inhibit access of targets to the complementary probes (Binder et al., 2004). All virus probes had at least one secondary structure with at least one hairpin, only 11.44% of virus probes had only one secondary structure. The Spearman correlation test showed a moderate positive relationship ($r=0.580$, $P<0.01$), between free energy and the number of folding, indeed, free energy increases with number of folding. Considering that the minimum free energy confers stability to bound base pairs, the opposite is thus true; when the free energy tends towards positive values; the DNA strand adopts non-stable bindings leading to a higher number of elementary structures.

Secondary structures of single-stranded DNA can be predicted by computing the minimum folding energy that represents the stability of the folding of the sequence (Xia et al., 2010). Several conformations were possible with some probes showing up to 12 molecular arrangements, where hairpins were the most encountered structure. However, the occurrence of a particular structure follows the Boltzmann distribution that showed that for all probes, the structure that was more likely to appear corresponded to the conformation with a minimum free energy.

The significance is that secondary structure formations prevent hybridisation of probes to their targets, Scherr et al. (2000), demonstrated that to be accessible for hybridisation, strands should be composed of more than a 10 nt

consecutive sequence stretch that is not involved in loops, bulges, joint sequences or a free end. Conversely, Riccelli et al. 2001 designed probes with a hairpin of 16 bp duplex and a 5 base loop with a capture length of 32 bases and compared hybridisation profiles with linear probes. Their results demonstrated that target capture by hairpin probes was faster and thermodynamically more stable than by linear probes.

Although the secondary structure and thermodynamic parameter predictions were computed for free single strands in a solution, a microarray is a completely different system that involves tethered monomers on a surface and with a free strand in solution. However, many authors agree that the thermodynamics can be a good criterion to rank probe candidates even though they approve its approximation (Lemoine et al., 2009). In fact, in a previous study, Fotin et al. (1998) showed that there was a positive correlation between ΔGs of duplexes on a chip and in solution.

In terms of hybridisation predictions, two approaches have been carried out, one based on binding free energy of probes and targets, and other on a software that predicts the percentage of hybridised probe to the target in the form of fluorescence intensity represented by intensity of yellow colour.

The use of the nearest-neighbour model to predict probe-target stability seems to be an accurate tool to be utilised during the design of short and long probes (SantaLucia, 1998; Kibbe, 2007; Gharaibeh et al., 2010).

Thermodynamically, ΔG of probe-target duplexes was distributed between

-7.1 to -72 kcal/mol, the difference not being significant when free energies were ordered into categories.

Probe-target mismatches have been studied mainly to understand the effect of mismatches on hybridisation signal intensity. One mismatch in a hybridised long probe-target complex does not prevent the development of signal intensity but will result in a significant lower signal than for a perfect match duplex (Gharaibeh et al., 2010). Eventually more mismatches are more likely to induce a less intense fluorescence signal. In this study, 28.9% of probes matched perfectly their targets and a higher proportion ($p<0.001$) was represented by mismatched duplexes (71.7%). In solution, duplexes with mismatches tend to be less stable than their perfectly matched duplexes (Fish et al., 2007) with free energies tending toward positivity. The Spearman correlation test showed that free energies and the number of mismatches of the duplex were strongly positively correlated ($r=0.943$, $p<0.001$), meaning that the more mismatches are present in the formed duplex, the higher is the predicted free energy value and consequently the less stable is the duplex.

Moreover, mismatches were defined according to their position in the duplex whether at the 3' terminal, 5' terminal or positioned centrally. The impact of their location on the binding free energies of the duplex was analysed by linear regression, which showed clearly that mismatches affect the stability of the probe-target duplex, but are more detrimental when they are in the central area of the duplex. These results are in conformity with previous studies where the smaller destabilising effect of mismatches situated in the probe ends was

observed compared to the central area of the probe (Letowski et al., 2004, Wick et al., 2006). Thus, long probes with mismatches in the central region are expected to bind more likely to non-target sequences (He et al., 2005). Others have stated that 18 or more random mismatches of 60-mer probes reduced hybridisation signal to background level (Hughes et al., 2001). Moreover, others have indicated that when mismatches are evenly distributed throughout the probes, lower hybridisation signal intensities were reported than when mismatches are randomly distributed (Deng et al., 2008). In fact, the hybridisation process starts in the segments situated at the ends of the probe. However, the fragments will remain bound for a shorter time compared with fragments towards the centre of the probe (Jayaraman et al., 2007). For optimal results, Poulsen et al. (2008) proposed to design 60-mer probes such that the section close to the surface has a higher binding strength than the central and distal part, nevertheless it is important to bear in mind that binding to targets is not a definitive situation as perfect match and mismatch are constantly formed during a hybridisation reaction (Dufva et al., 2009).

In silico hybridisation equilibrium prediction between probes and targets by ChipCheck showed a relatively high negative correlation ($p<0.01$) with free energy of binding of PEDV probes to their respective targets. However, there was no relationship between experimental and predicted signal intensities for PEDV. Similarly, the three parameters tested were not correlated for the other virus probes. The negative correlation of free energy and predicted signal intensity seems consistent with the hybridisation dynamics of two

complementary strands. In effect, the free energy of a closed system tends to adopt a state of formed DNA base pairs duplex when the free energy is minimal (Linko and Dietz, 2013). The results in the present study show that the effectiveness of predicted free energies cannot be associated with signal intensities, consequently it seems realistic to conclude that predictions of probe hybridisation are inaccurate; however these findings resulted from testing only for five viruses and 38 probes. A higher number of probe hybridisation results, in conjunction with a more controlled hybridisation environment, should provide a more suitable substance to perform reliable relationship comparison. Also in this study the hybridisation of probes to their corresponding targets only were predicted. A simultaneous computation of probes and targets of several organisms would allow the estimation of the extent of cross-hybridisation, as well as the reliability of the application. The use of a prediction tool prior to hybridisation assay is a useful mean to out-distance properly the experiment so that only the best probes can be utilised in the assay. Besides the cost engaged in the fabrication of the platform and spotting of the probes can be reduced reliably as the microarray will be only subjected to few optimisations regarding the hybridisation conditions and not the probe specificity and sensitivity.

This programme may assist in microarray design by predicting its theoretical performance (Siegmund et al., 2003). The advantages and disadvantages of each of these software in terms of sensitivity and specificity will be covered in the final discussion (Chapter 7).

Chapter 4. Pathogen Detection Using PCR

4. 1. Introduction

Diagnosis of infectious agents in pathological samples from diseased individuals involves detection of molecular markers such as whole genes or gene sequences. PCR is a technique that allows the amplification of a specific fragment of DNA using a pair of primers that recognise complementary sequences in a mixture of extracted nucleic acid. Multiplex PCR is a simultaneous detection of several targets using a set of specific primers in a single reaction. Both PCR and multiplex PCR have already been shown to be a good method to detect pathogens causing diarrhoea (Platts-Mills et al., 2012; Sjöling et al., 2015).

Reference and clinical samples are a valuable resource for testing the detection methods used in the laboratory. They also offer the opportunity to optimise the assay allowing greater accuracy. For instance the use of designed primers and optimisation of the annealing temperature are both important in the success of the amplification.

In this chapter PCR reactions (monoplex and multiplex) were used to identify pathogens in known reference samples and in diarrhoeic samples from cattle and pigs. This was done for pathogens for which reference samples were available. Also, In this study, monoplex PCR was regarded as the gold standard method.

PCR (multilplex followed by monoplex) detection was performed on clinical samples for initial screening purposes. Samples showing the presence of enteric pathogens by PCR were subjected to array hybridisation. The main reason of adopting this approach was that the microbial status of the samples was unknown, and for logistical reasons, this avoided the risk of processing negative samples. This is a practical and economic reason to limit the number of arrays used and their cost, because they are still expensive and we could not use them systematically for all samples.

The use of designed primers in multiplex PCR led to similar PCR product sizes, and multiplex analysis was thus followed by individual monoplex PCR reactions.

4. 2. Material and Methods

4. 2. 1. Samples

Total nucleic acid (DNA and RNA) was extracted together from bovine and porcine faecal samples. Porcine faecal samples were kindly supplied by Dr M. Le Bon, School of Biosciences (Chapter 2). Bovine faecal samples were sourced from farms in Algeria. Samples were shipped using separate preservatives for DNA and RNA (DNAStable and RNAStable, Biomatrica, USA).

Nucleic acid was also extracted from reference strains (bacterial cultures, supernatant from infected cell cultures with viruses).

4. 2. 2. Co-extraction of DNA and RNA from faeces using RTP® Pathogen Kit

Nucleic acid extraction kits involve column purification that was developed to replace phenol / chloroform extraction techniques (Yang et al., 2011). Dual extraction of both pathogen RNA and DNA from biological samples necessitates specific chemistries to provide the maximum recovery of each nucleic acid.

The principle of the Ready-To-Prep (RTP) kit (Stratec Molecular) is based on sample lysis in a chemical environment at three different temperatures (with lyophilised lysis buffer and proteinase K in the lysis tube), followed by binding of nucleic acids to the spin filter. All reagents were supplied with the kit. Two successive washings to remove contaminants and ethanol were then performed with final elution of the nucleic acids.

Processing of faecal samples was performed according to Hofmann et al. (2012) (adapted for the first time from use with human stools). Using a sterile loop, a portion of faecal sample was picked and mixed with 500 µl of PBS. The solution was vortexed and centrifuged at 11,000 x g for 5 min. A volume of 160 µl of VXL Qiagen lysis buffer was mixed with 40 µl of the supernatant to which 200 µl of Resuspension Buffer were added. The mixture was transferred into an extraction tube L (lysis tube) and subjected to three consecutive incubations in a thermomixer at different times and temperatures (10 min at 37°C, 10 min at 65°C and 8 min at 95°C). The binding step consisted of the addition of 400 µl of binding solution followed by vortexing. The mixture was loaded in the RTA spin filter set and incubated at room temperature for 1 min, then centrifuged at

11,000 x g for 2 min. The spin filter was placed in a new tube after discarding the filtrate and 500 µl of wash buffer R1 were added to the spin filter. The spin filter with the tube was centrifuged at 11,000 x g for 1 min. A new tube was placed under the spin filter to perform a second washing with 700 µl of wash buffer R2 with centrifugation at 11,000 x g for 1 min. The filtrate was discarded and the spin filter dried by centrifugation at maximum speed for 4 min. The spin filter was placed in a 1.5 ml microcentrifuge tube to carry out the elution by adding 55 µl of preheated (to 65°C) elution buffer and incubation for 3 min at room temperature. Co-purified nucleic acids were recovered after centrifugation at 11,000 x g for 1 min and stored at -20°C.

4. 2. 3. Preparation of extracted nucleic acid for transport at room temperature

Only extracted nucleic acid using the RTP Pathogen kit was subjected to room temperature transport. Eluted total nucleic acid (60 µl) was divided into two equal volumes in two separate 1.5 ml microtubes. 7.5 µl of DNAStable and 20 µl of RNAStable were added to the DNA and RNA tubes, respectively. The contents of each tube were mixed by pipetting, and then completely dried overnight in a laminar hood at room temperature (22-25°C). The tubes were placed in moisture-barrier foil bags supplied with the kit with the accompanying desiccant packet (silica gel) and sealed for storage and transport.

4. 2. 3. 1. Sample recovery

Samples in DNAStable and RNAStable were stored at room temperature for a period of 15 to 30 days. To recover nucleic acid from the samples, 30 µl of nuclease free water (Qiagen) were added to each tube, mixed by pipetting and left at room temperature to rehydrate for approximatively 15 min. After recovery, all the samples were stored at -20°C until use.

4. 2. 4. Extraction using QIAamp® DNA Stool Mini Kit

A pea size (about 200 mg) sample of faecal matter was homogenised in 1.4 ml of Buffer ASL supplied with the kit, vortexed for 1 min than heated in a thermomixer (BioShake iQ – Quantifoil Instruments GmbH) for 5 min at 70°C. Faecal particles were then pelleted by centrifugation in a bench centrifuge at full speed for 1 min and 1.2 ml of the supernatant was transferred in a new tube. Removing faecal inhibitors was carried out by adding one InhibitEX tablet to the mixture and vortexing to form a suspension, which was then incubated for 1 min at room temperature. The tubes were centrifuged at full speed for 3 min and the supernatant transferred to a new tube, which was centrifuged once again at full speed for 3 min. A volume of 200 µl of the supernatant was mixed with 15 µl of proteinase K and 200 µl of buffer AL and incubated at 70°C for 10 min, then 200 µl of absolute ethanol were added to the mixture. The complete lysate volume was transferred to the spin column placed in a collection tube and centrifuged at full speed for 1 min. The filtrate was washed twice, first with 500 µl of AW1 (both AW buffers supplied with the kit) and centrifuged at full speed for 1 min, then a second washing was performed with

500 µl of AW2, the tubes were centrifuged at full speed for 3 min. The spin column was centrifuged once again with a new collection tube at full speed for 1 min. DNA was eluted twice in 60 µl of buffer AE, after incubating for 1 min at room temperature and centrifuging the spin column at full speed for 1 min, it was then stored at -20°C until used.

4. 2. 5. Viral RNA Mini Kit (Qiagen)

This technique was carried out only for BVDV RNA extraction from a sample of medium supernatant containing BVDV. A volume of 560 µl of lysis buffer AVL was added to 140 µl of supernatant followed by pulse-vortexing for 15 seconds and incubated at room temperature for 10 min. The tube was centrifuged briefly and then 560 µl of ethanol and the tube mixed vigorously and briefly centrifuged. The binding step was performed by applying 630 µl of the solution to a mini column then centrifuged at 6000 x g for 1 min - the filtrate was discarded and a new tube was placed. This latter step was repeated once more. The column was washed with 500 µl of buffer W1, centrifuged at 6000 x g for 1 min and the collection tube was discarded. The column was washed once again but with 500 µl of buffer W2 followed by centrifugation at full speed (20,000 x g) for 3 min. The column was then dried by centrifuging at full speed for 1 min followed by the collection tube being discarded and replaced by a new one. Elution was carried out by adding 40 µl of buffer AVE and incubating the tube at room temperature for 1 min followed by centrifugation at 6000 x g for 1 min. To increase the RNA yield, this later step was repeated.

4. 2. 6. Genomic DNA extraction from tissue

Sections from small intestine wall and rectal wall were obtained from a specified pathogen free (SPF) pig (kindly supplied by Dr Dan Tucker, Cambridge Veterinary School). DNA extraction was carried out using a DNA Mini Kit (Qiagen). Approximately 25 mg of intestine wall tissue were cut aseptically into small pieces, to which 180 µl of buffer ATL and 20 µl of proteinase K were added. The mixture was briefly vortexed and incubated at 56°C for 210 min in a thermomixer at 600 rpm. A volume of 200 µl of lysis buffer AL was then added and mixed by pulse-vortexing for 15 seconds. The tube was incubated at 70°C for 10 min, then 200 µl of ethanol were added, mixed for 15 seconds and briefly centrifuged. The lysate was then applied to a mini column and centrifuged at 6000 x g for 1 min. The filtrate was discarded after the tube was placed in a new microcentrifuge tube. Washing step consisted of adding 500 µl of buffer W1 and centrifugation at 6000 x g for 1 min. A second washing was performed with 500 µl of W2 followed by centrifugation at 20,000 x g for 3 min. At each step the filtrate with the collection tube were discarded and a new tube was placed. Finally, the total DNA was eluted in 200 µl of buffer AE that was incubated at room temperature for 1 min, centrifuged at 6000 x g for 1 min and stored at -20°C.

Nucleic acid was quantified in all samples using using a NanoDrop8000 (Thermo Scientific™).

4. 2. 7. Sequence-specific amplification

4. 2. 7. 1. Monoplex PCR

PCR reactions were performed in a 50 µl final volume. RNA samples were subjected to reverse transcription for cDNA synthesis (detailed in section 5.2.3 – Chapter 5). The master mix comprised 35.5 µl of water, 1.5 µl of 50mM $MgCl_2$, 5 µl of 10 x Mg-free buffer, 0.5 µl of 25mM dNTPs, 0.5 µl of Taq polymerase, 5 µl of cDNA or DNA sample, 1 µl of 10µM forward primer and 1 µl of 10µM reverse primer (Appendix II and IV).

For the confirmatory detection of pathogens in clinical samples, primers published previously or designed in this study were used. PCR cycling conditions and PCR product length are illustrated in Appendix III. A negative control sample was included in each assay. PCR products (5 µl) were examined by electrophoresis in 1% agarose stained for fluorescence with Nancy-520 (10%). The mixed infections in bovine and porcine samples were performed using monoplex PCR reactions.

4. 2. 7. 2. Multiplex PCR

Reactions were set up using a QIAGEN® Multiplex PCR Plus kit in a final volume of 20 µl. Ten microliters of 2x Multiplex PCR Master Mix were mixed with 2 µl of 10x primer mix (2µM of each primer), 6 µl of nuclease free water and 2 µl of template (DNA, cDNA). The kit was designed for parallel detection with multiple primers and without optimisation. Two cycling conditions were used regardless of annealing temperature of each pair of primers (Table 4.1) for

amplicon size of up to 500bp and up to 1.5kb. In both situations, reactions were carried out for 40 cycles.

Table 4.1. Cycling conditions protocol for multiplex PCR

Cycling phase	Amplicon size up to 500 bp	Amplicon size up to 1.5 bp
Initial denaturation	95°C – 5 min	95°C – 5 min
Denaturation	95°C – 30 sec	95°C – 30 sec
Annealing	60°C – 90 sec	60°C – 90 sec
Extension	72°C – 30 sec	72°C – 90 sec
Final extension	68°C – 10min	68°C – 10min

4. 2. 8. Verifying the identity of donated reference samples

Monoplex PCR reactions were performed for 16 species of pathogens to confirm their identity (see 4.2.7.1)

Multiplex PCR was also carried out with reference strains (see 4.2.7.2) prior to hybridisation onto the array. Designed primers (Appendix IV) were used in both reactions.

4. 2. 9. Screening of samples (reference and clinical) with published and designed primers

The literature was searched for published primers targeting pathogens producing enteritis in cattle and pigs (Table 3.2 - Chapter 3). Primers were designed in order to flank a sequence in the pathogen that covers a maximum number designed probes. Amplicons resulting from PCR from designed primers were expected to have a size of less than 2 kb. All primer sequences were synthesised and desalted by Sigma-Aldrich, with a synthesis scale of 0.05 µmole.

Monoplex PCR reactions were performed for reference strains and clinical samples using designed primers, while multiplex reactions were carried out using published and designed primers, independently. Chronologically, clinical samples were first screened with published primers, then with designed primers in a multiplex PCR setting and finally with designed primers in monoplex reactions.

The relatively high resolution and high number of multiplexing using the chip regardless of similar amplicon sizes obviates the need for electrophoresis and sequencing. The chip would act as a single platform for detection, identification and analysis.

Monoplex reactions were performed to test the specificity of designed primers for reference pathogen species. PCRs were carried out according to the protocol described in this chapter, using thermocycling conditions for 30 cycles as shown in table 4.2.

The genes targeted with designed primers were 16S rRNA for *S.* Enteritidis, *S.* Typhimurium, *C. difficile* (with *tcdA*) and *Brachyspira pilosicoli*, the 16S-23S intergenic spacer for *C. jejuni*, F4ac and *faeG* (F4 fimbrial major subunits for *E. coli* F4), the K99 fimbrial subunit for *E. coli* F5, 18S rRNA for *Eimeria acervulina* and *Cryptosporidium* spp., NS1 for Porcine bocavirus, VP2 for Porcine parvovirus, VP7 for Porcine rotavirus, membrane, nucleocapsid, envelope and polyprotein for respectively primer pairs 1 and 3 for PEDV, capsid and the replicase for PCV2 and ORF1b for TGEV.

Table 4.2. Cycling conditions for monoplex PCR with designed primers

Pathogen	Cycling phase	Temperature - Time	No cycles
S. Enteritidis - *S.* Typhimurium- *C. jejuni- C. difficile* (16S and *tcdA*)- Por. bocavirus- Por. parvovirus- Por rotavirus – *Cryptosporidium* spp. – *E. coli* F4 – PEDV – PCV2- TGEV	Initial denaturation	94°C – 2 min	1
	Denaturation	94°C – 30 sec	30
	Annealing	60°C – 30 sec	
	Extension	72°C – 2 min	
	Final extension	72°C – 7 min	1
E. coli F5 *Brachyspira pilosicoli* *Eimeria acervulina*	Initial denaturation	94°C – 2 min	1
	Denaturation	94°C – 30 sec	30
	Annealing	57°C – 30 sec	
	Extension	72°C – 2 min	
	Final extension	72°C – 7 min	1

4. 3. Results

4. 3. 1. Reference sample identification

Twenty two known pathogen strains were kindly provided for this study either as bacteria, nucleic acid (DNA, cDNA or RNA) or faecal samples positive for

Bovine kobuvirus and Porcine torovirus. PCR reactions were performed as previously described and the results are illustrated in table 4.3.

4. 3. 1. 1. Published primers

A number of reference strains were tested with published primers using the cycling conditions shown in Appendix III. Primers targeted various genes: 18S rRNA for *Cryptosporidium* spp., VP7 for Porcine rotavirus, ORF1 for PCV, 16S rRNA for *C. jejuni*, *C. difficile* strains (630 and R20291), *S.* Enteritidis and *S.* Typhimurium, toxins A and B for *C. difficile*, *mdh* for *E. coli*, 5'UTR, Npro and capsid genes for BVDV (genotypes 1 and 2), spike gene for PEDV and TGEV, RdRp gene for Bovine kobuvirus, NS1 for Porcine bocavirus and porcine parvovirus and RdRp and capsid genes for Nebovirus. The appearance of a band on the gel after electrophoresis (Fig. 4.1, 4.2, 4.3, 4.4, 4.5) corresponding to the expected amplicon size was considered as a positive result confirming the identity of the strain (Table 4.3).

Table 4.3. Results of PCR reactions for reference strains with published primers

Pathogen	Expected amplicon size (bp)	PCR result
Cryptosporidium spp (11 strains)	435	-
Porcine rotavirus A	1062	±
PCV 1/2	646	+
E. coli F5	304	+
S. Enteritidis	574	+
S. Typhimurium	574	+
C. jejuni	857	+
C. difficile 630	643 (*tcdA*)- 399 *(tcdB*)- 900 (16S rRNA)	+ + +
C. difficile R20291	643 (*tcdA*)- 399 *(tcdB*)- 900 (16S rRNA)	+ - +
BVDV ncp	1013	±
BVDV cp	1013	-
PEDV	650	+
TGEV	859	+
Bovine kobuvirus BV250	216	+
Bovine kobuvirus BV253	216	±
Porcine bocavirus	680	+
Porcine parvovirus	265	+
Nebovirus	1669	-

± : Doubtful

The gel electrophoresis image in Fig. 4.1 did not reveal the expected amplification for *Cryptosporidium*, while Fig. 4.2, 4.3, 4.4 and 4.5 showed DNA bands at the expected molecular weight for PCV1/2, *E. coli , S.* Enteritidis, *S.* Typhimurium, *C. jejuni, C. difficile* 630, *C. difficile* R20291, PEDV, TGEV, Bovine kobuvirus BV250, Porcine bocavirus and P. parvovirus. Results for all strains of *Cryptosporidium* showed an incorrectly low molecular weight band and with two strains showing a clear smearing, BVDVcp, and Nebovirus. Multiple bands were observed for Porcine rotavirus A, BVDVncp and Bovine kobuvirus 253.

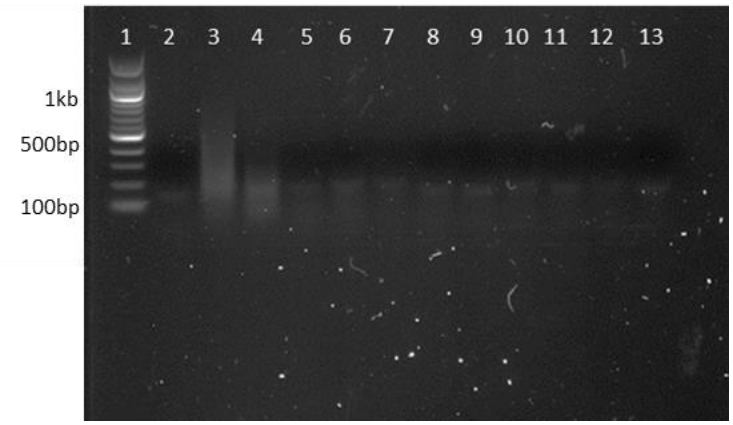


Figure 4.1. Agarose gel electrophoresis of specific amplification of *Cryptosporidium*.

1: Ladder (100 bp), 2: Negative control, 3: strain 19135, 4: strain 19136, 5: strain 19205, 6: strain 19270, 7: strain 19293, 8: strain 19310, 9: strain 19327, 10: strain 19330, 11: strain 19333, 12: strain 19334, 13: strain 19346. The size of the expected PCR product was 435bp.

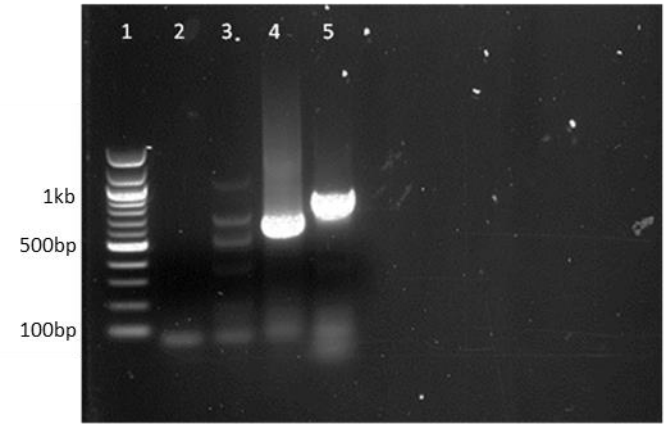


Figure 4.2. Agarose gel electrophoresis of specific amplification of Porcine rotavirus, PCV2 and *Campylobacter jejuni*.

1: Ladder (100 bp), 2: Negative control, 3: Porcine rotavirus, 4: PCV2, 5: *Campylobacter jejuni*. Only two strains show a band on the gel (PCV2 and C. jejuni with amplicon size of 646bp and 857bp respectively). P. rotavirus (lane3) showed non specific multiple band on the gel, with the top band corresponding to the expected amplicon size (1062bp).

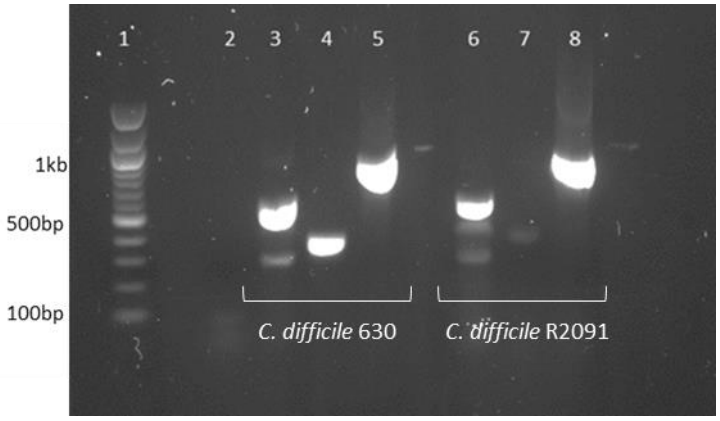


Figure 4.3. Agarose gel electrophoresis of specific amplification of *Clostridium difficile*.

1: Ladder (100 bp), 2: Negative control, 3: *C. difficile* 630/*tcdA*, 4: *C. difficile* 630/*tcdB*, 5: *C. difficile* 630/16S rRNA, 6: *C. difficile* R20291/*tcdA*, 7: *C. difficile* R20291/*tcdB*, 8: *C. difficile* R20291/16S rRNA. *C. difficile* 630 was positive for *tcdA*, *tcdB* and 16S rRNA, *C. difficile* R2091 was positive for *tcdA* and 16S rRNA with the expected amplicon sizes.

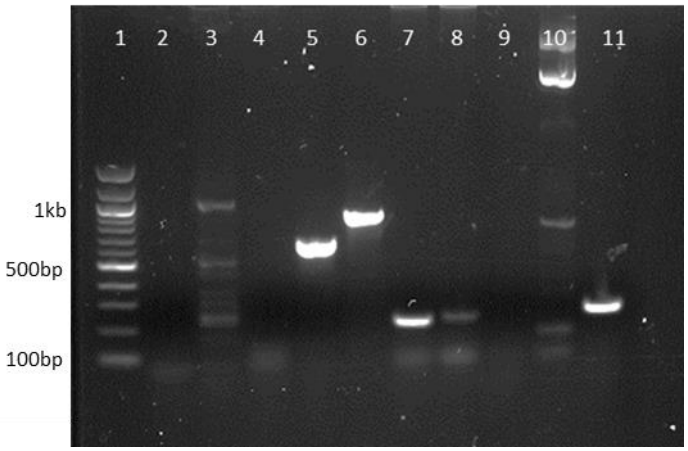


Figure 4.4. Agarose gel electrophoresis of specific amplification of BVDV (ncp and cp), PEDV, TGEV, Bovine kobuvirus, Nebovirus, Bocavirus and parvovirus

1: Ladder (100 bp), 2: Negative control, 3: BVDVncp, 4: BVDVcp, 5: PEDV, 6: TGEV, 7: Bovine kobuvirus BV250, 8: Bovine kobuvirus BV253, 9: Nebovirus, 10: Porcine bocavirus, 11: Porcine parvovirus. Expected amplicon size were obtained for PEDV, TGEV, B. kobuvirus and P. parvovirus (lane 5, 6, 7 and 11), also a faint band can be observed in lane 8 for B. kobuvirus but with the expected product size. Non specific PCR bands were generated by BVDVncp and P. bocavirus primers (lane 3 and 10), whereas no product was observed for BVDVcp and Nebovirus (Lane 4 and 9).

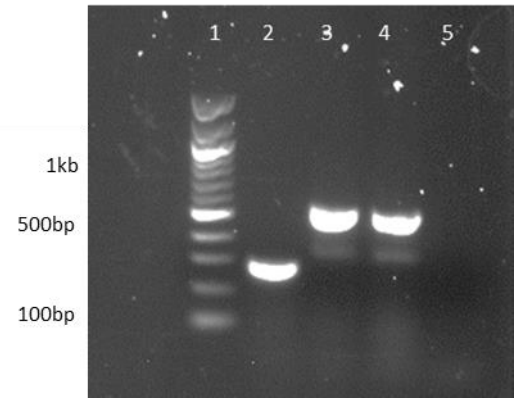


Figure 4.5. Agarose gel electrophoresis of specific amplification of *E. coli*, *S.* Enteritidis and *S.* Typhimurium

1: Ladder (100 bp), 2: *E. coli* F5, 3: *S.* Enteritidis, 4: *S.* Typhimurium, 5: Negative control. E. coli and the two serovars of Salmonella showed the expected amplicon size of 304 bp and 574 bp respectively.

4. 3. 1. 2. Designed primers

Designed primers were tested by PCR on 12 pathogen species including *S.* Enteritidis, *S.* Typhimurium, *E. coli* F4, *E. coli* F5, *C. difficile* (strain 630 and R20291), *C. jejuni*, *B. pilosicoli*, *Eimeria acervulina*, Porcine parvovirus, Porcine bocavirus, Porcine rotavirus A, PEDV, PCV and TGEV. The PCR results are summarised in table 4.3. The gels showed that 13 out of 15 reactions were in agreement with the expected product size (Fig. 4.6 and 4.7), whereas amplification of Porcine parvovirus and Porcine bocavirus genes resulted in non-specific products that did not match the correct expected amplicon size

Table 4.4. Results of PCR reactions for reference strains with designed primers

Pathogen	Expected amplicon size (bp)	PCR result
S. Enteritidis	1500	+
S. Typhimurium	1492	+
C. jejuni	965	+
E. coli F4	760	±
E. coli F5	1120	+
C. difficile 630	1469 (16S rRNA)- 1437 (*tcdA*)	+
C. difficile R20291	1469 (16S rRNA)- 1437 (*tcdA*)	+
B. pilosicoli	1624	+
E. acervulina	1244	+
Porcine parvovirus	1158	-
Porcine bocavirus	1264	-
Porcine rotavirus A	521	+
PEDV (using 2 primer pairs)	1928 (PEDV-1) – 1647 (PEDV-3)	+
PCV2	543	+
TGEV	1388	+

± : Doubtful

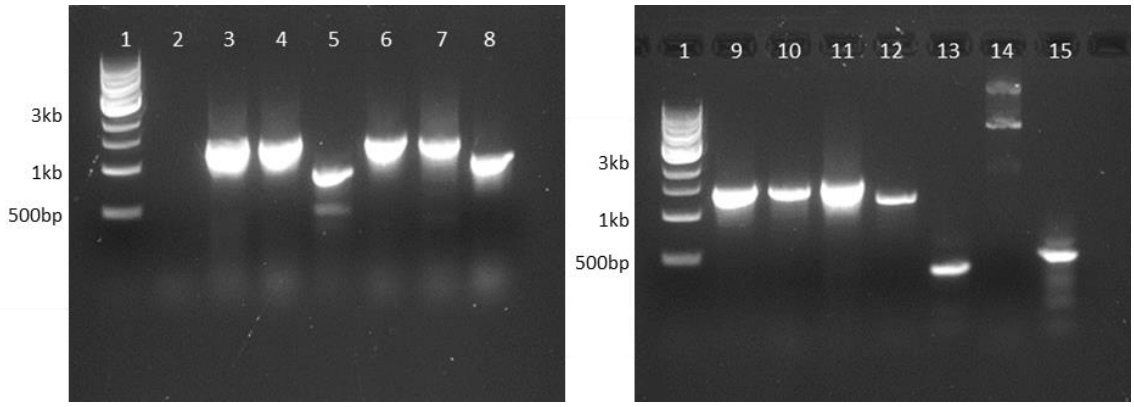


Figure 4.6. Agarose gel electrophoresis of specific amplification of *S.* Enteritidis, *S.* Typhimurium, *E. coli* F5, *C. difficile* (strain 630 and R20291), *C. jejuni*, *B. pilosicoli*, *E. acervulina*, Porcine parvovirus, Porcine bocavirus and Porcine rotavirus.

1: Ladder (1 kb), 2: Negative control, 3: *S.* Enteritidis, 4: *S.* Typhimurium, 5: *C. jejuni*, 6: *C. difficile* R20291 16S rRNA 7: *C. difficile* R20291 *tcdA*, 8: *E. coli* F5, 9: *C. difficile* 630 16S rRNA, 10: *C. difficile* 630 *tcdA*, 11: *B. pilosicoli*, 12: *E. acervulina*, 13: Porcine parvovirus, 14: Porcine bocavirus, 15: Porcine rotavirus A. All amplified PCR products had the expected size, except from P. parvovirus (lane13) and P. bocavirus (lane 14) which should be 1158 bp and 1264 bp respectively.

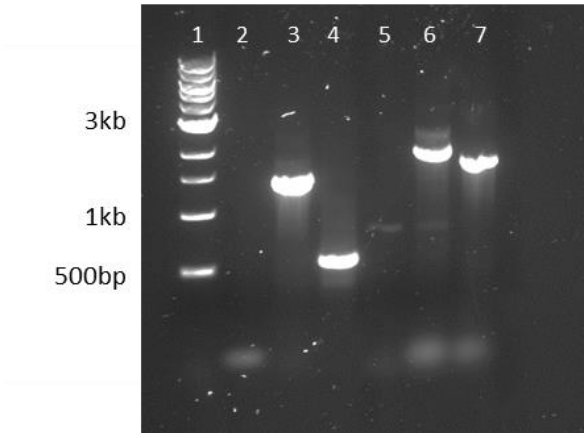


Figure 4.7. Agarose gel electrophoresis of specific amplification of PEDV, PCV and TGEV

1: Ladder (1kb), 2: Negative control, 3: TGEV, 4: PCV2, 5: *E. coli* F4, 6: PEDV-1, 7: PEDV-3. Lane 3, 4, 6 and 7, indicate the presence of the size of the PCR products. *E. coli* F4 amplification produced a faint band on lane 5, however with the expected amplicon size.

4. 3. 2. Clinical sample screening

4. 3. 2. 1. Bovine samples

Bovine clinical samples (129, among which, 123 from calves), from Algeria were investigated for the presence of enteric pathogens with published and designed primers, five samples from camels and one from a goat were also included in this study. Among a total of 129 faecal samples, nearly 45% (n=58) were negative by multiplex PCR assays using published primers (Appendix X). Similarly, 42.63% (n=55) were negative for assays with designed primers. Also, 33 samples (25.58%) were negative by both assays.

a. Multiplex PCR

Results from the application of multiplex PCR with published and designed primers to bovine samples showed variation between the same samples in both reactions (Fig. 4.8, 4.9, 4.10 and 4.11) which led to performing array

hybridisations to clarify the findings. The numbered tracks on the agarose gel electrophoresis images represent the names of the samples.

One source of discrepancy was the product sizes which were very similar for a few species, for instance 304 bp and 281 bp for *E. coli* and Bovine Parvovirus, respectively, (indicated with an orange arrow – Figs. 4.8 and 4.10) and 1062 bp and 1013 bp for Bovine and Porcine rotavirus and BVDV (indicated with a yellow arrow- Fig. 4.8). Similarly, the molecular weight of *Yersinia* spp., Bovine coronavirus and Bovine enterovirus amplicons were almost equal to 400 bp (indicated with a white arrow – Figs. 4.8 and 4.10), which made gel reading difficult.

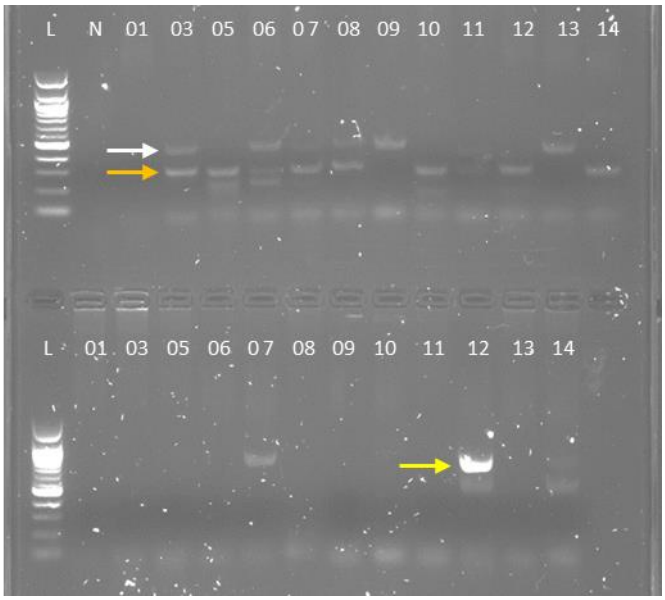


Figure 4.8. Agarose gel electrophoresis of pathogen detection in bovine samples with published primers by multiplex PCR - Batch 2

Upper lanes represent amplicon size of up to 500 bp and lower lanes represent amplicons size of up to 1.5 kb. L: Ladder (100 bp), N: Negative control, 01-14: Samples.Orange arrow indicates either the presence of *E. coli* with an amplicons size of 304 bp or B. parvovirus with an amplicon size of 281 bp. White arrow indicates an amplicons of 400 bp that correspond to *Yersinia* spp. or B. enterovirus. Yellow arrow indicates a PCR product of 1013 bp or 1062 bp corresponding to BVDV or B. rotavirus respectively.

The same drawbacks were faced when reading electrophoresis gels of amplified products with designed primers in which the majority had a size ranging from 1391 bp for Bovine enterovirus to 1447 bp for *Cryptosporidium* spp., to 1458 bp for *Salmonella* spp. and 1475 bp for Bovine rotavirus (indicated with a yellow arrow - Fig. 4.9). Another set of three pair of primers (Coronavirus, Kobuvirus and EPEC *eae*) resulted in the same conflicting results with respective expected amplicon sizes of 1617 bp, 1659 bp and 1569 bp (indicated with a white arrow – Fig 4.11) (Appendix XI)

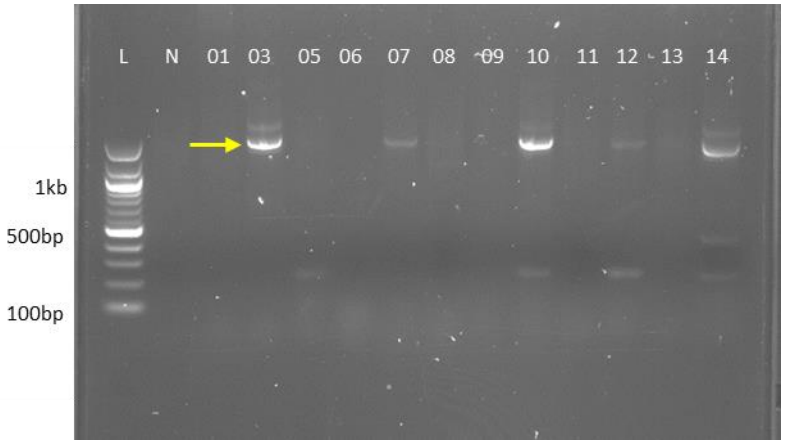


Figure 4.9. Agarose gel electrophoresis of pathogen detection in bovine samples with designed primers by multiplex PCR - Batch 2

All primers were designed to generate amplicons exceeding 1 kb. L: Ladder (100 bp), N: Negative control, 01-14: Samples. Yellow arrow indicates an amplicon of 1617 bp, 1659 bp or 1569 bp corresponding to B. coronavirus, B. kobuvirus or EPEC *eae* respectively.

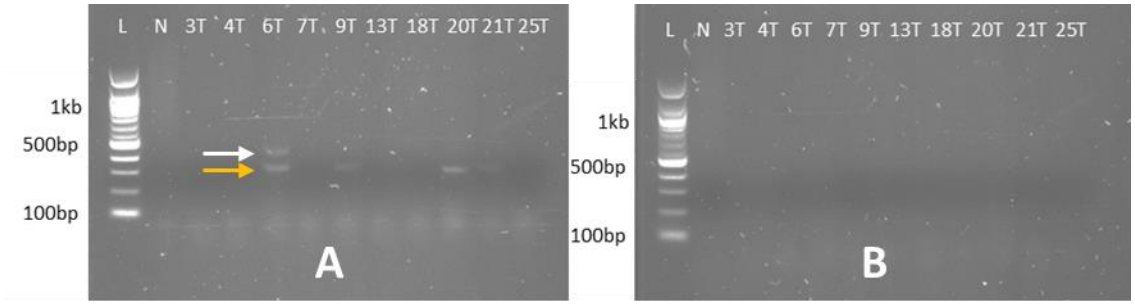


Figure 4.10. Agarose gel electrophoresis of pathogen detection in bovine samples with published primers by multiplex PCR- Batch 4

A: Less than 500 bp PCR product size, B: Up to 1.5 kb PCR product size

L: Ladder (100 bp), N: Negative control, 3T- 25T: Samples. Orange arrow indicates either *E. coli* with an amplicons size of 304 bp or B. parvovirus with an amplicon size of 281 bp. White arrow indicates an amplicons of 400 bp that corresponds to *Yersinia* spp. or B. enterovirus.

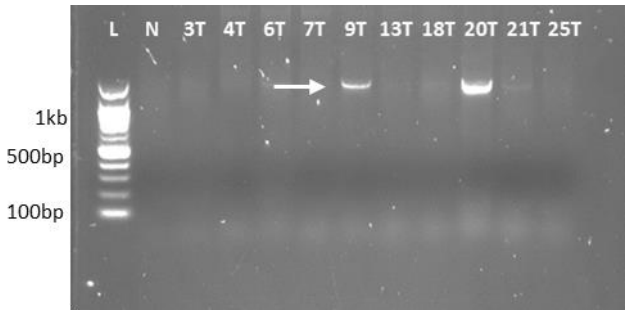


Figure 4.11. Agarose gel electrophoresis of pathogen detection in bovine samples with designed primers by multiplex PCR - Batch 4

L: Ladder (100 bp), N: Negative control, 3T - 25T: Samples. All primers were designed to produce amplicons size of more than 1kb. White arrow indicates four possible pathogens of 1391 bp (B. enterovirus), 1447 bp (*Cryptosporidium* spp.), 1458 bp (*Salmonella* spp.) or 1475 bp (B. rotavirus).

In this section, few representative gel electrophoresis images illustrate the pathogens detected by multiplex PCR using published and designed primers in the same samples.

b. Monoplex PCR

The results from monoplex reactions with designed primers showed that 25.58% (n=33) of samples were positive, with 74.42% (n= 96) negative to the targeted pathogens. *Cryptosporidium* spp. and *E. coli* F5 were detected

relatively frequently with 40.91% and 38.64%, respectively. BVDV was present in 11.36% of the positive samples, additionally the *E. coli* attaching effacing gene (*eae*) and Bovine kobuvirus were both detected in 4.55% of the total positive samples (Table 4.5).

Table 4.5. Detected pathogens in bovine samples

Pathogen	Number	Percentage (%)
Salmonella spp.	-*	-*
E. coli F5	17	38.64
E. coli eae	2	4.55
Cryptosporidium spp.	18	40.91
BVDV	5	11.36
Bovine kobuvirus	2	4.55
Total	44	100

* : Data removed for non-specificity of Salmonella primers

With regard to mixed infections, which were all detected by monoplex PCR (Fig. 4.12), 25% (n= 11) of a total number of 44 positive samples contained at least two targeted enteropathogens (they could be distinguished by separate bands on the gel by monoplex and multiplex PCR using designed primers) with 16S rRNA of *Salmonella* spp. present in nearly all positive samples. The highest co-infection rate was represented by *E. coli* F5 and *Cryptosporidium* spp. (81.82%, n=9 of positive samples), *E. coli* F5 and BVDV, and by *E. coli eae* and *Cryptosporidium* spp. which showed 9.09% (n= 1) each.

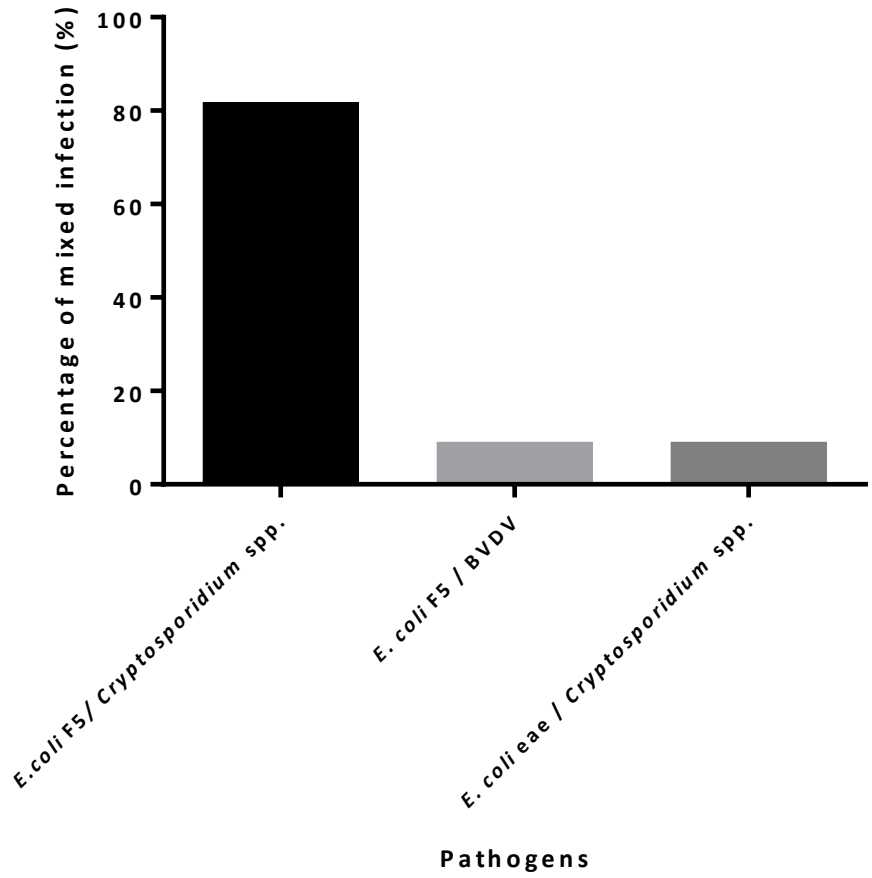


Figure 4.12. Distribution of mixed infection in bovine faecal samples
More than 80% of mixed infections were with *E. coli* F5 and *Cryptosporidium* spp. Other mixed infections (*E. coli* - BVDV and *E. coli* – *Cryptosporidium*) were present in 9% only.

2.4.1.1. Porcine samples

Porcine samples were kindly donated by Dr M. Le Bon who conducted a study on the use of probiotics in diarrhoeic piglets that have died during the first week of their life. These samples had been already examined for the presence of *C. difficile* and its toxin A and B and Porcine rotavirus A by enzyme immunoassay by Le Bon's team. A series of monoplex and multiplex PCRs were applied to 34 porcine faecal samples, which tested positive either for *C. difficile*,

C. difficile A and B toxins, Rotavirus A or all of them. Six additional samples were included because they were negative by ELISA. Table 4.6 and 4.7 illustrate the monoplex and multiplex PCR results respectively.

Table 4.6. Monoplex PCR results using published (P) and designed (D) primers

Pathogen	Total of tested samples	No	%
Porcine rotavirus A (P)	34	2	5.88
Porcine rotavirus A (D)	45	7	15.55
Porcine kobuvirus (D)	45	12	26.67
C. difficile 16S (D)	43*	-	-

*: The number of *C. difficile* positive using 16S rRNA were ignored due to the lack of specificity of the primers.

Table 4.7. Multiplex PCR results using designed primers

Pathogen	Total of tested samples	No	%
Negative	40	14	35
C difficile 16S		16*	-
C difficile tcdA		1	2.5
C difficile tcdB		9	22.5
Porcine kobuvirus		5	12.5
Porcine rotavirus A		9	22.5

*: The number of *C. difficile* positive using 16S rRNA were ignored due to the lack of specificity of the primers.

The monoplex PCR results (Appendix XII) showed that only 5.88% (2 out of 34) of the samples contained Porcine rotavirus A when PCRs were carried out with published primers, while 15.55% (7 out of 45) were positive when assayed with designed primers. Only one sample result produced using published primers was in agreement with the PCR using designed primers.

Multiplex PCRs were performed with designed primers (Appendix IV).These showed that 9 (22.5%) of 40 samples were positive for *C. difficile* based on the presence of *tcdA* and *tcdB* genes. All these samples contained *tcdB* and only

one was *tcdA* positive. Rotavirus and kobuvirus were detected with respective rates of 22.5% (n=9) and 12.5% (n=5).

Monoplex reactions were able to detect the presence of kobuvirus with higher rates than multiplex reactions, with the exception of rotavirus where the same rate of detection was observed.

Screening samples by multiplex PCR (n= 40) showed evidence of mixed infections (Fig. 4.13). A frequency of 17.5%% (n=7) of infected samples was attributed to mixed infections. The most frequently encountered co-infections were *C. difficile* with rotavirus (3 samples, 43%), C*. difficile* with kobuvirus (2 samples, 14%), rotavirus and kobuvirus (1 sample, 14%) and *C. difficile* with rotavirus and kobuvirus (1 samples, 14%). The multiplex was also able to detect mono-infection where 5 samples (26.32%) were infected with *C. difficile* and rotavirus alone and 2 (10.53%) were infected with kobuvirus alone.

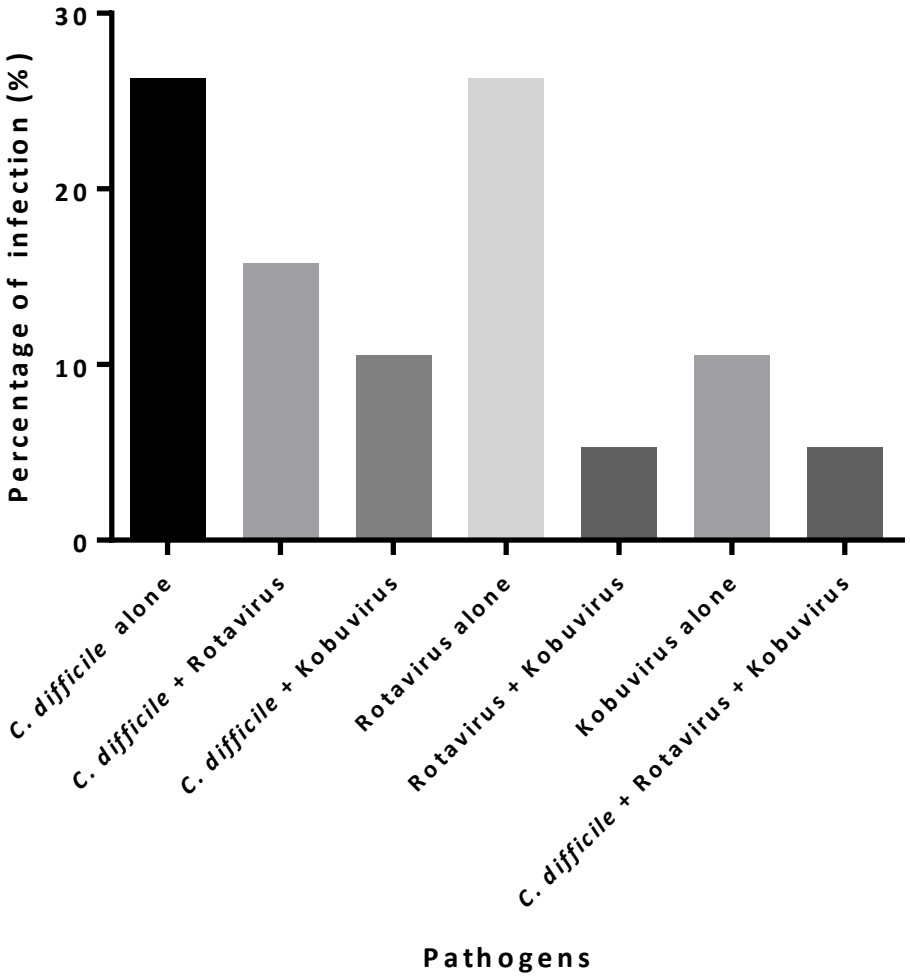


Figure 4.13. Distribution of mono-infections and co-infections in 19 porcine faecal samples by multiplex PCR

The main pathogens found were *C. difficile* and rotavirus which were both detected alone in 26.32%. *C. difficile* was in association with viruses in 31.58%. Mixed infections were detected in 36.84% of the positive samples.

4. 4. Discussion

For this project, published and designed primers were used to amplify target genes for two main reasons, namely (i) to verify the identity of pathogens in the donated reference samples and (ii) to assess the specificity of designed primers. This was done for those pathogens for which reference samples were available and was thus not an exhaustive study.

Although not all the same reference strains were tested evenly with designed and published primers, the majority of the strains were detected successfully with both categories of primers indicating the value of the primers for analysis of the clinical samples by PCR. PCR reactions using published, but not designed, primers were performed for *Cryptosporidium* strains, BVDV genotypes 1 and 2, Bovine kobuvirus strains and Nebovirus. *B. pilosicoli* and *E. acervulina* were done with designed primers only. The Porcine rotavirus VP7 gene was amplified only using designed primers whereas published primers used to amplify the same target generated multiple bands despite applying the recommended PCR conditions. Likewise, the NS gene of Porcine bocavirus showed a positive amplification with published primers, which was not the case for designed primers targeting the same gene.

With some exceptions all strains showed a positive amplification with the correct product size according to the known expected molecular size of amplicons. However, a few strains exhibited a multiband pattern in the gel, which can be caused by multiple annealing of the primers to the DNA resulting in several amplicons of different molecular weight. Non-specific amplification

is frequently the result of reduced annealing temperature (Lorenz, 2012). Also no optimisation was carried out.

Unlike most of the other pathogens no amplification was observed for *Cryptosporidium* strains, Porcine parvovirus and Nebovirus, which might be due to the absence of DNA, a very low amount or degraded DNA due to improper shipment conditions/storage during shipment. Two *Cryptosporidium* samples produced smears on agarose gel of 435 bp. This is not surprising as these samples were unfortunately held up during shipment and arrived late without dry ice.

Amplification of porcine parvovirus with designed primers generated an unexpected band of less than 500 bp (the desired product size was 1158 bp) which can also be attributed to mispriming, this might have been be due to low temperature stringency (Hecker and Roux, 1996), again despite using the recommended/published PCR conditions.

Bovine clinical samples were screened for the presence of major enteric pathogens with the purpose of identifying samples with which initial array analyses could be carried out. The multiplex PCR with published and designed primers showed that a number of pathogens were detected in reactions with the published, but not the designed primers and vice-versa with almost the same frequencies, 15.50% and 17.05% respectively. However, multiplex reactions with both types of primers were able to detect genes in 39.53% of the total number of samples. The multiplex PCR results of both assays were not conclusive, as the PCR product sizes were almost comparable. Thus, the rate of

agreement between the two assays was not calculated and consequently, microarray experiments were used to differentiate between targeted genes and further confirm the presence of a specific pathogen. The reasons for the absence of amplicons using published primers could conceivably be due to typographical errors in published primer sequences, or more practically due to different reagents and equipment used to perform the PCR although this remains to be demonstrated.

The amount of extracted DNA and RNA from clinical samples was not indicative of high or low sensitivity or specificity of the PCR assays, as highly DNA/RNA-rich samples did not necessarily result in the amplification of targeted genes. This however, was largely dependent on the specificity of the primers and on the qualitative aspect of the DNA. For example, PCV-2, a donated reference virus, was detected using a pair of designed primers despite an amount of 5 ng of DNA/µl. The clinical samples had a nucleic acid amount range of 41-129 ng/µl for DNA and 34-100 ng/µl for RNA.

Monoplex PCRs were carried out using designed primers only. Initially, the results showed that *Salmonella* spp. was identified in 72.87% of samples. A BLAST search of the forward and reverse primer sequences (16S rRNA) showed that they not only allowed amplication with *Salmonella* spp. but also with other related members of the *Enterobacteriaceae*, such as *E. coli* 16S rRNA, *Enterobacter* spp. 16S rRNA, *Klebsiella pneumoniae* 16S rRNA and *Serratia* spp. 16S rRNA. Primer-Blast claims to check the specificity of the primers by testing the target-specific primers against several databases that include RefSeq mRNA

database, RefSeq genome database and nr database. However, to prevent time-consuming operations of the system, instead of checking the specificity of each candidate primer, the software performs BLAST search for the entire submitted query sequence that was submitted once for a BLAST search (Ye et al., 2012), which clearly could have produced misleading results. A specificity checking online tool, MFE primer-2.0 (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/) (Qu et al., 2012), that takes into consideration the specificity of both entered primers simultaneously and their ability to produce amplicons, was utilised to explain the presence of high proportion of *Salmonella* spp. in bovine faecal samples (data not shown). This programme generated 1336 potential amplicons of 1458bp, with 780 amplicons for *E. coli* and 326 for *S. enterica* and *S. bongori*, probably explaining here why things went wrong and how that was addressed. In theory, a primer design tool should exclude primers that would amplify non-target sequence. In this study, PCR products amplified by *Salmonella* spp. primers might therefore not be the expected amplicons; they could be *Salmonella* spp. or *E. coli*. Moreover, knowing the abundance of *E. coli* in the intestine compared to *Salmonella*, it is highly probable that the amplified PCR products were *E. coli*, either commensal or pathogenic as this was identified in most samples. Thus, proportion calculations of pathogens in samples were done without presumptive *Salmonella* identification, and the same approach was applied to *Clostridium difficile* identification in porcine faecal samples. Among a total of 94 positive samples, 44 were strains of bacteria, viruses and parasites.

Cryptosporidium spp and *E. coli* F5 were detected in 41% and 39% of samples, respectively despite the difficulty in deciding on how far the *Cryptosporidium* assessment was valid. Various studies reported lower rates for *Cryptosporidium* infections in calves; in Algeria, a prevalence of 17% to 25% was reported (Khelef et al. 2007; Ouchene et al., 2012). In other countries almost similar general figures have been described (Swai et al., 2007; Maurya et al., 2013; Qi et al., 2015), while others have recorded lower proportions varying from 6% to 12% (Mallinath et al., 2009; Khan et al., 2010). However in the former, the detection technique of oocysts was by Sheather's sugar floatation (Sheather, 1923) instead of molecular identification. Although confirmation of a similar rate of isolation and thereby frequency of the pathogen does not necessarily indicate the validity of the test. It is encouraging that following *in vitro* evaluation results were obtained, which in all probability reflects the true prevalence in the field. There seems no doubt that a molecular approach, if the assay could be refined, would be a major step forward in accurate diagnosis.

E. coli F5 is a major infectious agent in new-born calves (Martín et al., 2003). In this study, 39% of positive samples contained *E. coli* F5, with lower prevalence values reported in other studies varying from 5% to 10% (Younis et al., 2009; Nguyen et al., 2011; Shams et al., 2012) and others recording higher rates more in line with the results of the present study (Shahrani et al., 2014). Achá et al. (2004) identified F5 fimbrial *E. coli* in both diarrhoeal and healthy calves, but with a higher prevalence in calves with diarrhoea. Pathotypes EPEC

(Donnenberg and Kaper, 1992), STEC (Gannon et al., 1993), EHEC (Tzipori et al., 1995) and AEEC (Pearson et al., 1999) have all been reported to contain the *eae* locus. The attaching effacing phenotype is genetically determined through the locus of enterocyte effacement (LEE) (McDaniel and Kaper, 1997). Intimate attachment to the intestinal epithelial cells surface causing attaching-effacing lesions is characterised by the formation of pedestals (Paton and Paton, 1998; Goosney et al., 1999). The *eae* gene has been detected in 2.13% of positive samples with higher results obtained in other positive studies fluctuating from 10 to 32% (Nguyen et al., 2011; Blanchard, 2012; Andrade et al., 2012; Shahrani et al., 2014).

Mucosal disease is a fatal condition of BVDV infection due to the superinfection of persistently infected calves with a cytopathic strain (BVDV genotype 2). Calves can be infected with non-cytopathic BVDV through vertical transmission. Once infected, they become persistently infected and shed and excrete the virus for lifetime. Animals are generally clinically asymptomatic, however a super-infection with cytopathic BVDV leads to fatal mucosal disease. Brownlie (1990) reported that BVDV has an immunosuppressive effect on calves, which increases their susceptibility to other enteric pathogens, contributing to the apparition of enteritis.

Among positive samples, 11% contained BVDV-2 using designed primers, considerably low prevalence rates were reported by the study of O'Connor et al. (2007) where animals were tested for BVDV with an antigen capture ELISA. A more recent study using PCR, identified virulent strains circulating in

European cattle herds with a prevalence of 9.5% (Schirrmeier, 2014), which is within the same range of values compared with our probably tentative figure. In America, where genotype 2 is highly prevalent, a Brazilian serological survey reported 32% - 42% of BVDV infected calves (Fernandes et al., 2016).

In this study bovine kobuvirus was detected by PCR with a low frequency (4.55%), which is in contrast with a particularly higher prevalence, that has been reported by Barry et al. (2011), where 77.8% of young calves were infected. Others have reported a molecular detection of 6% to 17% (Yamashita et al., 2003; Khamrin et al., 2008; Reuter and Egyed, 2009; Jeoung et al., 2011). Kobuviruses have also been described in healthy animals, however their presence at a higher rate in animals with diarrhoea (Khamrin et al., 2008) suggested that they might play a role in intestinal infections in young calves. In addition, it has been shown that infections with the virus is age related, and predominated in calves under the age of 1 month (Khamrin et al., 2008; Jeoung et al., 2011). Clearly more needs to be done here ideally with a reference sample to validate the test before an assessment of the isolation frequency from diseased and healthy animamls can be carried out accurately.

It is important to consider the contribution of presumably mildly pathogenic organisms in mixed infections as unknown interactions may occur between pathogens leading to an exacerbation of the symptoms with aggravation of the condition. This has been demonstrated with human patients infected simultaneously with HIV and *M. tuberculosis* (Pape et al., 1993) and which has been suitably called a "danger-couple model" (Shankar et al., 2014). PCR is an

accurate and sensitive tool that can allow the detection of the presence of several pathogens in a clinical sample (Reller et al., 2007) although multiplexing can limit discrimination where amplicons may be similar sizes thus emphasising the need to ensure where possible that this is not the case. High throughput technologies such as microarray (or NGS through metagenomics) have the capabilities to allow a more extensive approach and permit inclusion of all possible aetiological agents in the identification process irrespective of amplicon size.

Virulence markers are important indicators of the arsenal of the causal pathogen present in the sample because they can be easily related to the symptoms observed during the development of the disease. Further to the acquired broad knowledge in terms of virulence and antibiotic resistance genes, the availability of their sequences in public databases allows oriented and customisable pathogen identification through the design of a core genome and broad pangenomic sets of oligonucleotides for an accurate microarray detection or for simple use with PCR assays. These genes should differentiate between two species with identical 16S rRNA, such as *gyrA* or *gyrB* (gyrase A or gyrase B), *tuf* (elongation factor Tu), *sodA* (manganese-dependent superoxide dismutase) and heat shock proteins (Reller et al., 2007).

A truly multiplex approach offered by microarrays would seem to be the best way forward for studying mixed infections which may contain combinations of any of the major or minor pthogens associated with enteritis.

Isolation rates differ between studies and seem to be influenced by the country where the study was conducted, farm management, hygiene and at the scale of the laboratory, they might vary upon sampling and used detection techniques. This small study using PCR rather than microarray indicates that mixed infections are likely to be a common occurrence, in this case a combination of *C. difficile*, Rotavirus and Kobuvirus in pigs and *E. coli*, BVDV and possibly *Cryptosporidium* in Algerian calves.

Multiple infectious agents have been implicated in calf diarrhoea (Cho et al., 2010). Among positive monoplex samples, 25% were mixed infections involving *E. coli* F5/ *Cryptosporidium* spp. (81.82%), *E. coli* F5/ BVDV (9.09%) and *E. coli eae*/ *Cryptosporidium* spp. (9.09%). Several studies reported a rate of mixed infections varying from 15% - 58% (Snodgrass et al., 1986; Abraham et al., 1992; de la Fuente et al., 1998; García et al., 2000). In the present study, it clearly appears that *E. coli* is present in association with viral or parasitic enteropathogens. However, the detection rate seems to be the highest for *E. coli* / *Cryptosporidium* spp., which is likely to be due to the high detection rate of these two enteropathogens, as found similarly in the study of de la Fuente et al. (1999) with the frequency of concurrent infection with *E. coli* F5 and *Cryptosporidium* spp. being 27.8%. An investigation conducted in Algeria in 2011 showed that calves co-infected with *E. coli* F5 and *Cryptosporidium* spp. represented the second most frequent mixed infections after rotavirus and *Cryptosporidium* (Akam et al., 2011). Indeed the most common reported co-infection by authors elsewhere was *Cryptosporidium* together with rotavirus

(de la Fuente et al., 1999; García et al., 2000; Akam et al., 2011). We hypothesised two reasons that can attribute the lack of detection of rotavirus, (i) efficiency of designed primers used for the identification of bovine rotaviruses. However, in this study designed specific primers to rotavirus were used in the diagnostic process with positive results. (ii) the nucleic acid extraction methods used could be one of the causes alongside problems with transport of preserved extracted nucleic acid. Together, these two situations could have had an impact on the amount of viral RNA in the samples suggesting that improving the transport of samples is an important factor to consider.

E. coli pathotype STEC possessing *eae* was also found in concomitant infection with *Cryptosporidium* spp. with a frequency value of 9.09% and co-infections involving these two pathogens have also already been reported in calves (Moxley and Smith, 2010). Mixed infections with *E. coli* F5 and BVDV-2 have also been recorded in 9.09% of positive samples in this study. Concomitant infection with BVDV is known to exacerbate intestinal infection (Wray and Roeder, 1987; Baker, 1990). The severity of the disease is also significantly increased when several pathogens infect the animal (Moxley and Smith, 2010). In porcine faecal samples, 22.5% were positive to *C. difficile* toxins. Scouring due to *C. difficile* is very common in neonatal piglets (Norén et al., 2014), particularly when infected with toxigenic *C. difficile* strains (Steele et al., 2010) but has not been recognised in the UK as a major cause of pig enteritis thus far and more needs to be done. Our results showed that all nine samples identified as containing *C. difficile* possessed the *tcdB* gene, with one isolate possessing

both *tcdA* and *tcdB* genes. Toxin A (TcdA) and B (TcdB) are major virulence factors in *C. difficile* (Dubberke et al., 2011). However, in comparison with toxin B, which seems essential for pathogenicity, the role of TcdA has not been completely elucidated (Lyras et al., 2009). Recently it has been demonstrated that TcdB alone causes severe damage to the intestine as well as systemic organ dysfunction (Carter et al., 2015).

Rotavirus A was identified in multiplex and monoplex reactions, with a higher proportion in a multiplex setting (22.5% versus 15.55%), which was not the case for kobuvirus that was detected in 26.67% of samples by monoplex PCR versus 12.5% in multiplex. This could indicate a possible number of competitions for nucleotides, primers and polymerase between pathogens in a multiple target reaction (Dabisch-Ruthe et al., 2012). Some authors have showed a decrease of amplification efficiency of 1 to 3 log10 when amplifying one target in the presence of several competitive targets (McElhinney et al., 1995; Jackson et al., 1996). Rotavirus A is a very common and highly pathogenic cause of enteritis in human and pig populations (Papp et al., 2013). In this study 22.5% of assayed samples by multiplex PCR contained rotavirus A. In diarrhoeic samples from young pigs, several studies have shown that rotavirus A was the major pathogen found with high frequencies varying from 46% to 84% (Bora et al., 2009; Midgley et al., 2012; Marthaler et al., 2014a). Much lower prevalences have been cited in other studies from 4% to 27% (Wieler et al., 2001; Halaihel et al., 2010; Midgley et al., 2012; Pham et al., 2014), which are more in the range of the results in this study. Also, nearly all reports indicated that

rotaviruses have also been detected in healthy pigs, but at significantly reduced rates. Porcine kobuvirus has been identified in pigs with diarrhoea as well as healthy pigs (Khamrin et al., 2010) and seems to be very prevalent even in healthy animals with 87.3% of samples testing positive in asymptomatic pigs in one study (Dufkova et al., 2013), whereas another study showed that the Porcine kobuvirus infection was significantly correlated with the occurrence of diarrhoea with 84.5% of diarrhoeic pigs and 19.3% non-diarrhoeic (Park et al., 2010).

In this study, animals simultaneously infected with several pathogens counted for 17.5% of positive samples, among which 43% were represented by *C. difficile* with rotavirus and 29% with kobuvirus. *C. difficile* was present in all detected co-infections; Yaeger et al. (2002), reported that 50% of pigs were co-infected by *C. difficile* and rotavirus. Other authors have shown that lesions caused by other enteropathogens can enhance enteric colonisation by *Clostridium* (Songer and Uzal, 2005). Indeed the high proportion of this virus-bacteria association is likely to be due to the combination of their virulence mechanisms. It has been demonstrated that *in vitro* infection with rotavirus enhances bacterial invasiveness of MA-104 cells with enterobacteria such as *Salmonella*, *Shigella* and *E. coli* (Bukholm, 1988). When artificially infected with rotavirus, followed by EHEC a few days later, piglets developed a more severe enteric disease than when challenged with each agent separately (Tzipori et al., 1980).

Additionally, results in this study showed that infection by the trio *C. difficile*, rotavirus and kobuvirus represented 14% of mixed infections, in addition to the damaging consequences of both *C. difficile* and rotavirus, an additive pathological consequence could thus occur when kobuvirus is present. In fact, a recent study has shown that infected pigs with kobuvirus had specific intestinal lesions that led to gastroenteritis (Yang et al., 2015). It may thus be that combinations of pathogens have unique pathologies resulting from the combined effects of their virulence attributes.

Chapter 5. Design and Evaluation of Alere Microarray

5. 1. Introduction

In chapter 4, samples were identified by PCR with known pathogen content prior to their evaluation using the Alere array, which is presented in this chapter.

The Alere microarray, platform ArrayTube™ was used to detect enteric viruses in samples. It comprises 201 probes and is a virus only microarray, with three biotin markers (as internal controls). To assess the basic performance of the microarray for selected pathogens, several tests were performed. Specificity experiments, building on the data generated in chapter 4, aimed to show that the microarray was able to correctly detect known organisms, without identifying as false positive, organisms not present in any sample. Sensitivity testing was also carried out to identify the detection limit of the arrays, compared to PCR.

These array evaluations then led to the final step undertaken to identify enteropathogens in clinical diarrhoeic samples of piglets and calves.

Prior to hybridisations, random and specific amplification strategies were employed. The aim was also to compare the detection efficiency when using the two types of amplification with known reference strains.

In the specific amplifications, multiplex and monoplex PCR using samples identified as positive from chapter 4. were used to identify the presence of one

or multiple pathogens, either with reference strains or in clinical samples that were demonstrated to contain more than one pathogen.

5. 2. Material and Methods

5. 2. 1. Pathogen DNA extraction

Pathogen DNA was extracted from faecal samples using RTP®Pathogen kit. It was also extracted with QIAamp®DNA Stool Mini kit from faeces or QIAamp® UCP Pathogen Mini kit from broth cultures.

5. 2. 2. Viral RNA extraction using RNeasy Mini Kit (Qiagen)

This kit was utilised only for viral RNA extraction from Bunyamwera virus, Schmallenberg virus and Equine influenza A virus (H3N8).

Infected cultivated cell supernatants were used for the extraction of RNA from the viruses using the RNeasy Mini Kit (Qiagen). A 200 µl aliquot of supernatant was first mixed with 600 µl buffer RTL and vortexed for few seconds, then 800 µl of 70% ethanol were added and mixed with the lysate by pipetting. A volume of 700 µl was transferred to a spin column and centrifuged at 8000 x g for 15 sec and the flow-through was discarded. Washing was carried out by adding 700 µl of buffer RW1 to the spin column and centrifuging at 8000 x g for 15 sec and the flow through was discarded. The spin column membrane was then washed twice with 500 µl buffer RPE, and centrifuged at 8000 x g but using two different centrifuging times, 15 sec and 2 min respectively, and on both occasions the flow through was discarded. To dry the membrane, an additional

centrifugation was performed for 1 min at full speed. Finally the RNA was eluted twice using 35 µl of RNAse-free water for a total volume of 70 µl.

Following RNA extraction, cDNA was synthesised (see 5.2.3), which was then biotin-labelled involving random amplification (see 5.2.4).

5. 2. 3. Reverse transcription and first strand synthesis of RNA samples

Viral RNA was reverse-transcribed using random primers. Two to five microliters of template were mixed with 1 µl of 40 µM of the random pentadecamer primer A (GTT TCC CAG TCA CGA TCN NNN NNN NNN NNN NN) and 1 µl of 10mM dNTP mix (Life Technologies), and sufficient water was added to reach a total volume of 13 µl. The tubes were heated to 65°C for 5 min in the thermal cycler and kept on ice for 1 min. To each tube, 7 µl of a mix of 4 µl of 5x reverse transcriptase buffer (Life Technologies), 1 µl of 0.1M Dithiothreitol (DTT) (Life Technologies), 1 µl of RNase inhibitor (40U/µl) (Life Technologies) and 1 µl of Superscript III reverse transcriptase (200U/µl) (Life Technologies) were added. The mixture was incubated at 25°C for 5 min, followed by 1 hour at 50°C and the enzyme was finally inactivated by incubation at 70°C for 15 min.

5. 2. 4. Nucleic acid amplification and labelling

5. 2. 4. 1. Primer extension and second strand synthesis reactions (Round A)

a. For reverse transcribed RNA samples (cDNA)

Sample denaturation was performed in the thermal cycler at 94°C for 2 min followed by cooling to 10°C for 5 min during which the reaction was paused and 10 µl of a mixture of 1 µl of 10x Klenow buffer (Promega), 8.7 µl of water and 0.3 µl of Klenow polymerase (5U/µl) (Promega) were added and the reaction resumed by incubation at 37°C for 8 min. A second cycle was carried out by heating the samples at 94°C for 2 min and cooling down to 10°C for 5 min during which 1.2 µl of diluted Klenow were added (1:4 in water). Tubes were then incubated at 37°C for 8 min. For each set of reactions a negative (non-template) control was introduced.

b. For DNA samples

A total volume of 10 µl of a mix was prepared with 1 µl of primer A, 1 µl of 10x Klenow buffer and 2 to 5 µl of DNA sample. Three to six microliters of water were added as required. Tubes were heated to 94°C for 2 min in the thermal cycler and cooled to 10°C for 5 min during which the reaction was paused and 5.05 µl of a mixture of 0.5 µl of 10x Klenow buffer, 1.5 µl of 3 mM dNTP mix, 0.75 µl of 0.1M DTT, 1.5 µl of BSA (500 µg/ml), 0.5 µl of water and 0.3 µl of Klenow polymerase were added. The reaction was resumed for incubation at 37°C for 8 min. The tubes were heated to 94°C for 2 min and cooled to 10 °C for 5 min during which 1.2 µl of diluted Klenow were added (1:4 in water).

Tubes were then incubated at 37°C for 8 min. For each set of reactions a negative control was introduced.

DNA samples were either PCR products produced by specific or random amplification.

5. 2. 4. 2. Amplification of Round A product (Round B)

A master mix was prepared with 1.5 µl of 50mM $MgCl_2$ (Invitrogen), 5 µl of 10x Mg-free buffer (Invitrogen), 0.5 µl of 25mM dNTP mix, 0.5 µl of 100µM primer B (GTT TCC CAG TCA CGA TC), 0.5 µl Taq polymerase (5 U/µl) (Invitrogen) and 3 to 7 µl of round A product. Water was added for a total reaction volume of 50 µl. The PCR reaction was performed using the programme : denaturation at 94°C for 10 sec followed by 35 cycles of 30 sec at 94°C, 30 sec at 40°C, 30 sec at 50°C and 2 min at 72°C, with a final extension carried out at 72°C for 2 min. The PCR amplification product was verified by running 5 µl of amplicons on a 1% agarose gel containing Nancy-520 (10%) (Sigma-Aldrich).

5. 2. 4. 3. Direct biotin labelling (Round C)

Five microliters of round B product were mixed with 32.5 µl of water, 1.5 µl of 50mM $MgCl_2$, 5 µl of 10x Mg-free buffer, 5 µl of 10x Biotin (Roche) labelling mix (1.0mM dATP, dCTP, dGTP, 0.65mM dTTP and 0.35mM Biotin-16-dUTP), 0.5 µl of 100µM Primer B and 0.5 µl Taq polymerase, for a total reaction volume of 50 µl. A PCR reaction was carried out under the same conditions used for round B, but in this case for 25 cycles.

5. 2. 5. Array Tube hybridisation

Two hybridisation protocols were carried out. An In-house protocol which has been applied for labelled reference strains (PEDV, TGEV, PCV-2, Porcine parvovirus, Porcine rotavirus, Porcine bocavirus, Bovine kobuvirus, Porcine torovirus and BVDV). The second protocol was the Alere protocol which was adopted for all other hybridisations on array tubes, using the Hybridisation kit (Identibac- Alere).

5. 2. 5. 1. In house hybridisation protocol

The procedure involved incubation and mixing of liquids added and removed (by pipetting) subsequently. All incubations and mixing steps throughout the protocol were performed using a BioShake iQ thermomixer (Quatifoil Instruments GmbH). The image of the hybridised array was captured and analysed by the array reader ArrayMate (Alere Technologies GmbH).

- **Array conditioning**

The ArrayTube (AT) was first washed with 500 µl of water for 20 min at 30°C, 550 rpm then washed with 500 µl of pre-hybridisation buffer (5x SSC, 0.1% SDS, 4x Denhardt's solution) for 30 min at 50°C and 550 rpm.

- **Hybridisation**

An aliquot of labelled DNA (10 µl) was mixed with 90 µl of 1x hybridisation buffer (5x SSC, 1% SDS, 4x Denhardt's solution) and denatured at 95°C for 3 min on a thermal cycler (Applied BioSystems 2720), then kept on ice. The sample was transferred into the AT to hybridise for 30 min at 50°C and 550 rpm.

- **Post hybridisation washings**

After hybridisation, three consecutive washings were performed. The solution was removed from the AT and 500 µl of wash buffer 1 (1x SSC, 0.2% SDS) were added and incubated for 20 min at 50°C and 550 rpm. Buffer 1 was removed and 500 µl of wash buffer 2 (0.1x SSC, 0.2% SDS) were added and incubated for 20 min at 40°C and 550 rpm. A final wash was carried out at 30°C for 20 min and 550 rpm with 500 µl of wash buffer 3 (0.1x SSC).

- **Blocking**

After removing washing buffer 3, 100 µl of blocking solution (2% biotin-free milk in PBS containing 1% BSA and 0.1% Tween 20) were added and incubated at 30°C for 60 minutes with mixing at 300 rpm.

- **Conjugation**

Blocking solution was removed from the AT and 100 µl of the conjugation solution (Streptavidin Poly-HRP conjugate (Thermo Fisher Scientific) diluted 1:100 in blocking solution) were added. The incubation was carried out at 30°C and mixing at 300 rpm for 15 min.

- **Post-conjugation washings**

The conjugation solution was removed from the tube and three successive washings were performed using the same conditions as for post-hybridisation washes.

- **Precipitation staining and detection**

Washing buffer 3 was carefully removed from the AT and 100 µl of TMB/H_2O_2 staining solution, TrueBlue (TMB/H_2O_2 Solution), (Insight Biotechnology LTD)

were added and incubated at 25°C for 8 min without shaking. After incubation, the TrueBlue was removed and up to six tubes were scanned using the ArrayMate (Alere). Generated data consist of an image taken by the ArrayMate and text file format (.txt) with raw data signal data. The data were exported in an external storage device for further analysis.

b. Alere hybridisation protocol

Labelled round C products of the three viruses were hybridised to ATs using a hybridisation kit (Identibac, Alere Technologies GmbH, Jena - Germany) from Alere according to manufacturer's instructions, but with few modifications. All reagents were supplied in the kit.

- **Array conditioning**

The tubes were conditioned with 500 µl nuclease free water and incubated at 30°C for 5 min at 550 rpm in a thermomixer (BioShake iQ - Quantifoil Instruments GmbH). The water was then removed and 300 µl of hybridisation buffer C1 were added and the tubes were incubated at 55°C for 7 min at 550 rpm. After incubation the buffer was removed.

- **Hybridisation**

A volume of 10 µl of each labelled product was mixed with 90 µl of hybridisation buffer C1 for a final volume of 100 µl. A sample denaturation step was carried out at 95°C for 5 min on a thermal cycler and the tubes were then kept on ice. Hybridisation was performed in the thermomixer, the denatured sample was pipetted into the tube and incubated 60 min at 55°C at 550 rpm.

- **Washings after hybridisation**

The hybridisation solution was removed and two consecutive washings were carried out using 500 µl of washing buffer C2 at 45°C for 5 min at 550 rpm.

- **Streptavidin-horseradish peroxidase conjugation**

The washing buffer was removed and 100 µl of streptavidin-horseradish peroxidase conjugate solution (by mixing horseradish peroxidase conjugate 100x C3 and conjugate buffer C4 at a dilution of 1:100) was added. Incubation was carried out at 30°C for 10 min at 550 rpm.

- **Washings after conjugation**

Two rounds of washings were performed with 500 µl of washing buffer C5 and incubation at 30 °C for 5 min at 550 rpm. Finally the washing buffer was removed and discarded.

- **Staining and tube-scanning**

A staining step of bound horseradish peroxidase conjugate was done by pipetting 100 µl of pre-warmed (30°C) horseradish peroxidase subtrate D1 into the tube and incubation at 30°C for 5 min without shaking. The ArrayTubes were then scanned using the ArrayMate (Alere). Data generated were exported in an external storage device.

5. 2. 6. Specificity of ArrayTube microarray

The specificity of the array involved the organisms that were represented by the probes on the array (nine reference viruses: PEDV, TGEV, PCV2, PPV, BVDV, Porcine rotavirus A, Porcine bocavirus, Bovine kobuvirus and Porcine torovirus)

and organisms that are not targeted by the array (three reference viruses: Bunyamwera virus (B1), Schmallenberg virus (S) and Equine influenza A virus (H3N8)). Two viruses (TGEV and PCV2) were selected to examine the difference between random and specific amplification on detection performance using these two approaches.

The specificity of the AT microarray was also tested with products from two multiplex PCRs. Ten species of bacteria, viruses and parasites were amplified according to the method in Chapter 4 (4.2.7.2) using specific primers for reference strains of TGEV, PCV2, *S.* Typhimurium, *S.* Enteritidis and *C. difficile*, multiplexed in one reaction and *B. pilosicoli*, PEDV, Porcine rotavirus A, *Eimeria acervulina* and *Campylobacter jejuni* in another multiplex PCR reaction.

Cytochrome b gene was also detected by PCR in host genomic DNA extracted from healthy porcine intestine tissue and bovine faecal samples. The PCR products were amplified randomly and biotin-labelled (see 5.2.4.1), then hybridised to the ArrayTube to assess the extent of cross-hybridisation with genomic host DNA and to identify the host of the clinical sample. All assays were conducted in individual tubes.

5. 2. 7. Sensitivity of ArrayTube

Two viruses were selected to perform the sensitivity testing on the Alere Tube platform; Porcine circovirus (PCV-2), which is a DNA virus and Transmissible gastroenteritis virus (TGEV) which is an RNA virus. DNA and cDNA from these two viruses were two-fold serially diluted and an aliquot from each dilution was used for PCR detection of a sequence of 1388 bp of the TGEV polyprotein and

a sequence of 543 bp of the PCV-2 replicase gene using designed primers. PCR products from each dilution of the two viruses were analysed by electrophoresis on a 1% agarose gel. They were then subjected to biotin labelling (see 5.2.4) followed by hybridisation to the ATs (see 5.2.5) (Fig. 5.1). Templates of PCR reactions contained host porcine genomic DNA spiked with viral DNA/cDNA aiming at a ratio of 1% (v/v).

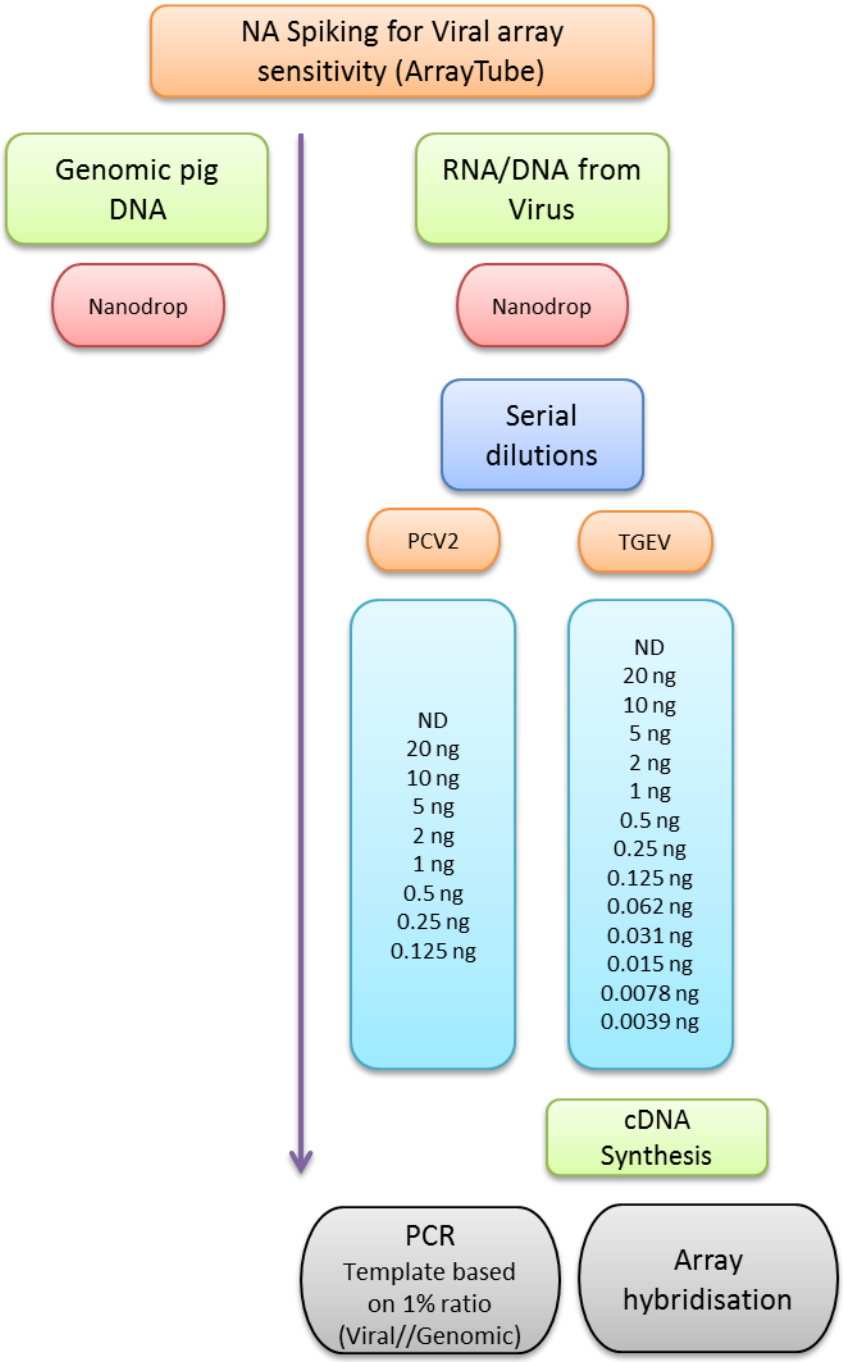


Figure 5.1. Illustration of the sensitivity protocol of ArrayTube
NA: Nucleic acid, ND: non-diluted

5. 2. 8. Hybridisation of clinical samples to Alere platform

5. 2. 8. 1. Porcine clinical samples

Four porcine clinical samples positive by PCR to Porcine rotavirus, Porcine kobuvirus and *Clostridium difficile* and its toxins (Table 5.1) were hybridised to ATs using amplicons from two different amplification approaches: random (see Round A – 5.2.4.1 and Round B - 5.2.4.2) and specific (Chapter 4 – 4.2.7.2) amplification. The specific amplification did not include *C. difficile* and *C. difficile* toxins, but only Porcine rotavirus A and Porcine kobuvirus were amplified using specific primers. In this multiplex PCR reaction both published and designed primers were utilised.

Table 5.1. Positive porcine faecal samples hybridised to Alere ArrayTubes – Results of multiplex PCR (Published and designed primers)

Sample	Published Pr. P.rotavirus A	Designed Pr. P.rotavirus A	Published Pr. P.kobuvirus	Designed Pr. kobuvirus
Sample 7	-	+	+	+
Sample 8	-	+	+	+
Sample 13	-	-	+	+
Sample 14	-	+	+	-

Designed Pr.: Designed primers, Published Pr.: Published primers

Another set of porcine positive samples by multiplex PCR including *C. difficile* and its toxins, Porcine rotavirus A and Porcine kobuvirus (Table 5.2) were hybridised to Alere Tubes (ATs).

Table 5.2. Positive porcine faecal samples hybridised to Alere ArrayTubes – Results of multiplex PCR (with designed primers only)

Sample	*C. difficile*16S rRNA	*tcdA*	*tcdB*	Porcine rotavirus A VP7	Porcine kobuvirus VP1
Sample 7	-	-	-	+	-
Sample 8	+	-	+	-	-
Sample 14	+	-	+	-	-
Sample C	+	-	+	+	-
Sample D	-	-	-	-	+
Sample G	+	-	+	+	+
Sample 4B	+	-	+	-	-
Sample 7B	-	-	-	-	+

The 9 samples described above were hybridised individually on ATs according to the protocol in 5.2.5 – b. Of note, PCR products were randomly amplified and biotin labelled.

5. 2. 8. 2. Bovine clinical samples

Twelve clinical samples were selected for hybridisation on AT platform based on their positivity to one of the targeted pathogens by multiplex PCR. Figures 5.2, 5.3, 5.4, 5.5 and 5.6 show the presence of PCR products using designed primers in all selected samples from chapter 4. It was not possible to distinguish the species of the amplified pathogen because molecular weights were nearly similar (1021 bp to 1659 bp) (Table 5.3). Sample 25T was negative to targeted species by multiplex PCR, but positive to BVDV by monoplex PCR (Fig. 5.2 and 5.5). This sample was selected to see whether the random amplification step prior to the biotin-labelling multiplex amplicon labelling) was able to amplify the viral genome when present, but not detected by multiplex PCR.

Table 5.3. Product size (bp) of amplicons with bovine designed primers

Pathogen	Product size (bp)
BVDV2_3	1073
Bovine kobuvirus	1659
Bovine enterovirus	1391
Bovine coronavirus	1617
Bovine rotavirus	1475
Bovine parvovirus	1021
Salmonella spp.	1458
ETEC F_5	1120
EPEC_eae	1569
Cryptosporidium spp.	1447

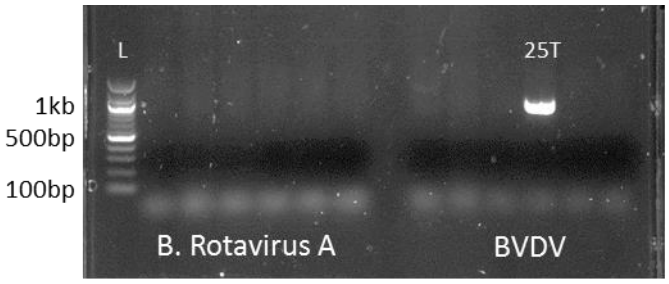


Figure 5.2. Agarose gel electrophoresis of Monoplex PCR – Bovine clinical samples

L: 100 bp DNA ladder, 25T: Positive sample – Batch 4, sample 25T was selected to be hybridised to the microarray as it was positive for BVDV, showing a PCR product size of 1073 bp.

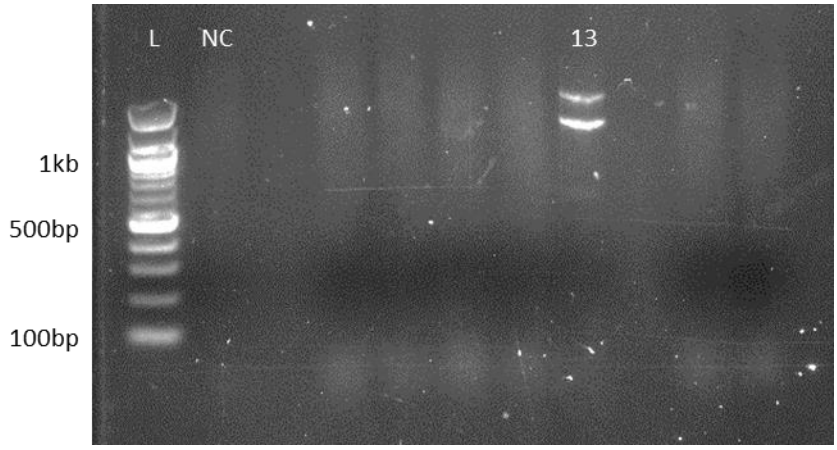


Figure 5.3. Agarose gel electrophoresis of Multiplex PCR – Bovine clinical samples

L: 100 bp DNA ladder, NC: negative control, 13: Positive sample – Batch 1, selected to be hybridised to the microarray

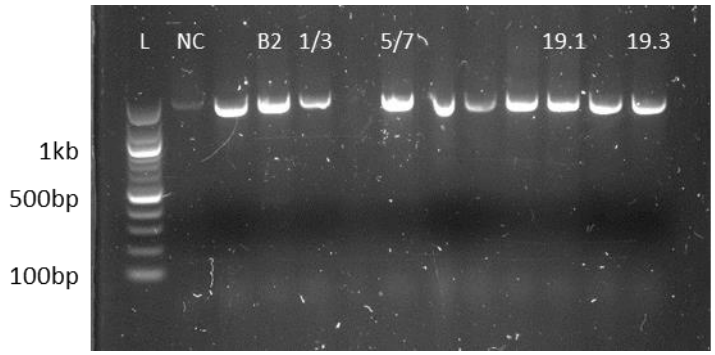


Figure 5.4. Agarose gel electrophoresis of Multiplex PCR – Bovine clinical samples

L: 100 bp DNA ladder, NC: negative control, B2 to 19.3: Positive samples (with bands on the gel) – Batch 3, samples B2, 1/3, 5/7, 19.0 and 19.3 were selected to be hybridised to the microarray.

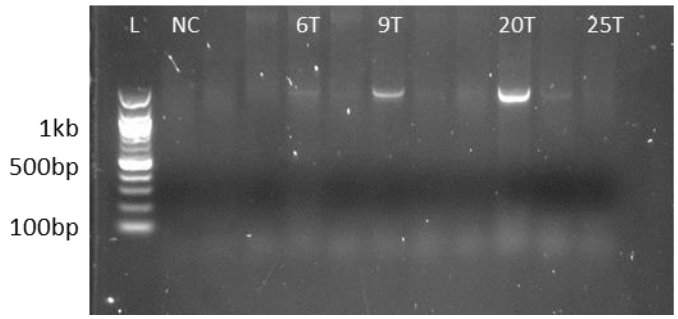


Figure 5.5. Agarose gel electrophoresis of Multiplex PCR – Bovine clinical samples

L: 100 bp DNA ladder, NC: negative control, 6T to 25T: Samples – Batch 4, samples 6T, 9T, 20T and 25T were selected to be hybridised to the microarray.

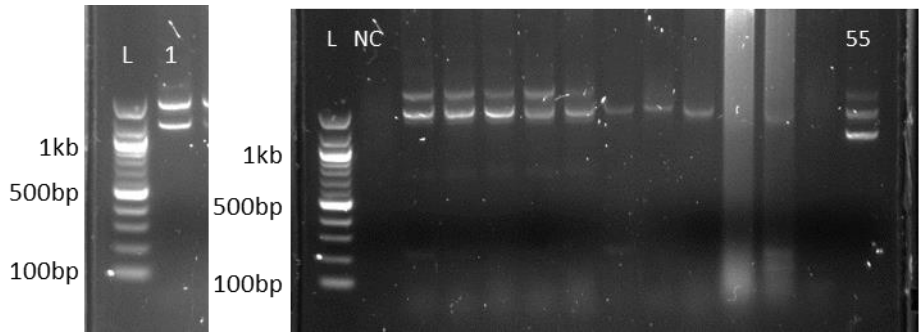


Figure 5.6. Agarose gel electrophoresis of Multiplex PCR – Bovine clinical sample

L: 100 bp DNA ladder, 1 and 55: Positive samples – Left image: Batch 6 with sample 1– Right image: Batch 9 with sample 55. Samples 1 and 55 were selected to be hybridised to the microarray.

All samples revealed on the previous gels were hybridised to ATs according to the protocol in 5.2.5 in this chapter.

5. 2. 9. Data analysis

Array images were analysed using Alere software IconoClust package which normalises the signal intensities for each spot by using the equation NI=1-(M/BG), where NI is the normalised intensity of a spot, M is the average

spot intensity and BG is the local background intensity. Normalisation output signals consist of values ranging between 0 and 1, where increasing values within the range indicate positivity.

For array hybridisation analysis, the threshold above which a sample is regarded as positive was calculated using the mean of background signal intensity plus three times the standard deviation. For known strains, the mean background intensity was calculated by averaging the highest 30 (Alere platform) or 50 (Agilent platform) spot signal intensities of non specific probes. For reference strains, probe intensities of viruses belonging to the same family/genus, were removed when the calculation of the limit of detection was performed, however they still appear on the figures.

Regarding clinical samples, cross-hybridising probes with the reference strains (ie non-specific probes with high signal intensity above the cut-off) were not considered in the calculation of the cut-offs. The background mean in this case was calculated by averaging all spots generating a signal above 0, leaving the highest signals of probes that showed a concordance with PCR findings as labelled amplicons were hybridised to the array, or formerly confirmed by PCR. The same approach was applied for the samples that were subjected to random amplification, except that in both situations a pattern is generally identified, where a number of specific probes correspond to the same pathogen.

Further to the array sensitivity tests of PCV and TGEV, a linear regression was computed to investigate the relationship between the amount of hybridised DNA and the signal intensity of the different probes using GraphPad Prism 7.01.

All spot intensities from each array were plotted in the figures presented in this chapter

5. 3. Results

5. 3. 1. Specificity and sensitivity testing of ArrayTubes

5. 3. 1. 1. Specificity of ArrayTubes with known strains

a. Array hybridisation with single reference strains

Reference strains, PEDV, TGEV, PCV-2, Porcine parvovirus, Porcine rotavirus A, BVDV, Porcine bocavirus, Bovine kobuvirus and Porcine torovirus were randomly-amplified, biotin-labelled and hybridised to the ATs. The hybridisation signal intensity for the viruses are illustrated in Figures 5.7 (PEDV), 5.8 (TEGV), 5.9 (PCV-2), 5.10 (PPV), 5.11 (P. rotavirus A), 5.12 (BVDV), 5.13 (P. bocavirus), 5.14 (B. kobuvirus), 5.15 (P. torovirus) and 5.16 (negative control).

Among the hybridised viruses, PEDV (Fig. 5.7), TGEV (Fig. 5.8), PCV-2 (Fig. 5.9) and PPV (Fig. 5.10) were correctly detected by the microarray with signal intensities for all or most of the spots higher than their respective threshold. Only two probes among 9 specific to BVDV hybridised for BVDV (Fig. 5.12). P. rotavirus A (Fig. 5.11), P. bocavirus (Fig. 5.13), Bovine kobuvirus (Fig. 5.14) and P. torovirus (Fig. 5.15) showed no hybridisation with the array with most or all of the pathogen-specific signals lower than the threshold. The negative control sample (Fig. 5.16) produced a pattern where all pathogen-specific probe signals were well below the cut-off point.

One spot (indicated by an arrow) representing probe Por_rotA_3 (Fig. 5.11) had an overall higher intensity in tested reference strains PEDV, PCV-2 and PPV exceeding the cut-off. In the negative control (Fig. 5.16), where nuclease-free water has been used as template, the signal intensity of this probe was 0.34. P. rotaA_3 spot remained visible in the other arrays, but nevertheless with a lower signal intensity,

One probe representing Sapovirus cross-hybridised with P. rotavirus A, also two probes representing Porcine norovirus viruses, although below the threshold showed relatively high signals (Fig.5.11).

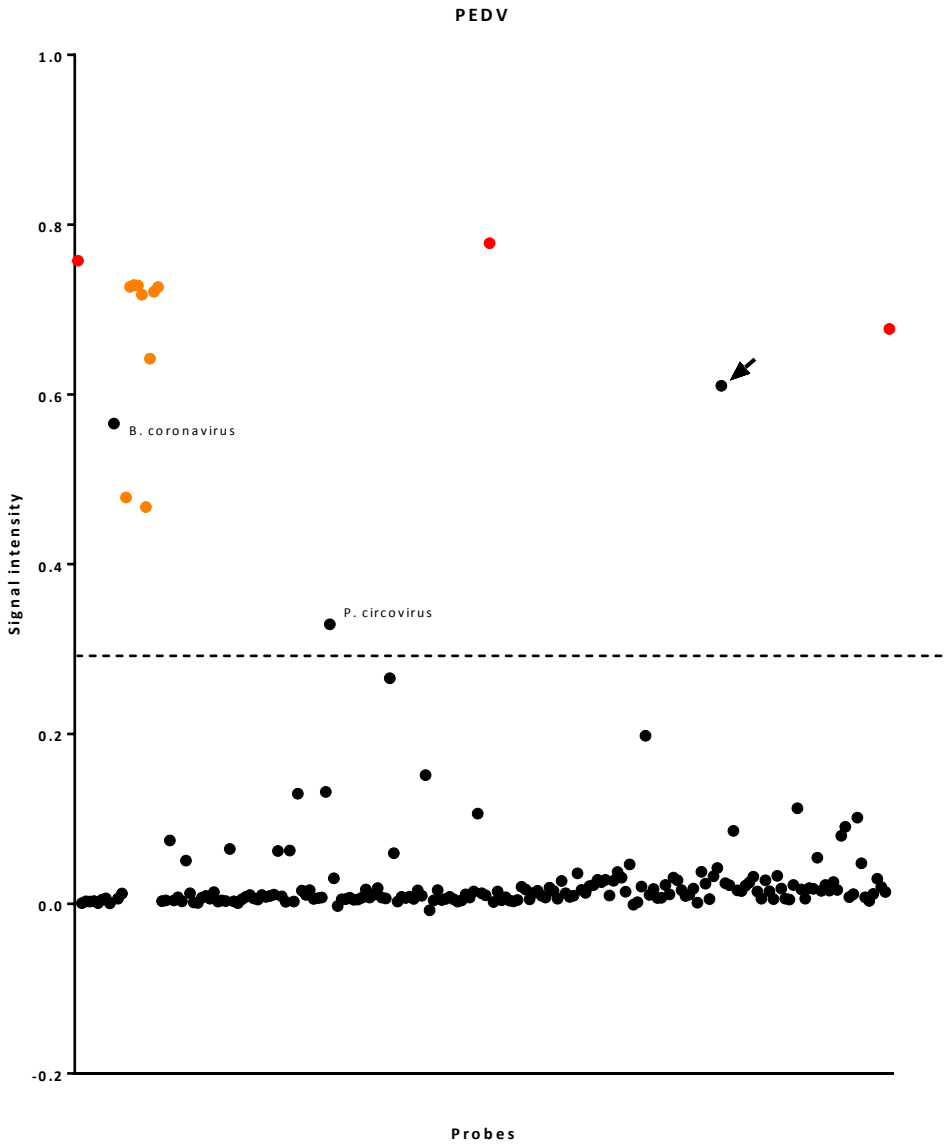


Figure 5.7. Normalised signal intensities of hybridised PEDV amplified by random amplification

Orange spots correspond to PEDV probes – Red spots correspond to Biotin markers. Arrow indicates Por_rotA_3. Signal cut-off of 0.29 is represented by a dashed horizontal line. PEDV strain showed positive hybridisation with all (n= 9) PEDV probes with signal intensity values higher than 0.29. One probe, Bov_cor_0227, had a signal intensity of 0.56 and was a coronavirus probe designed from the polyprotein ORF1a gene.

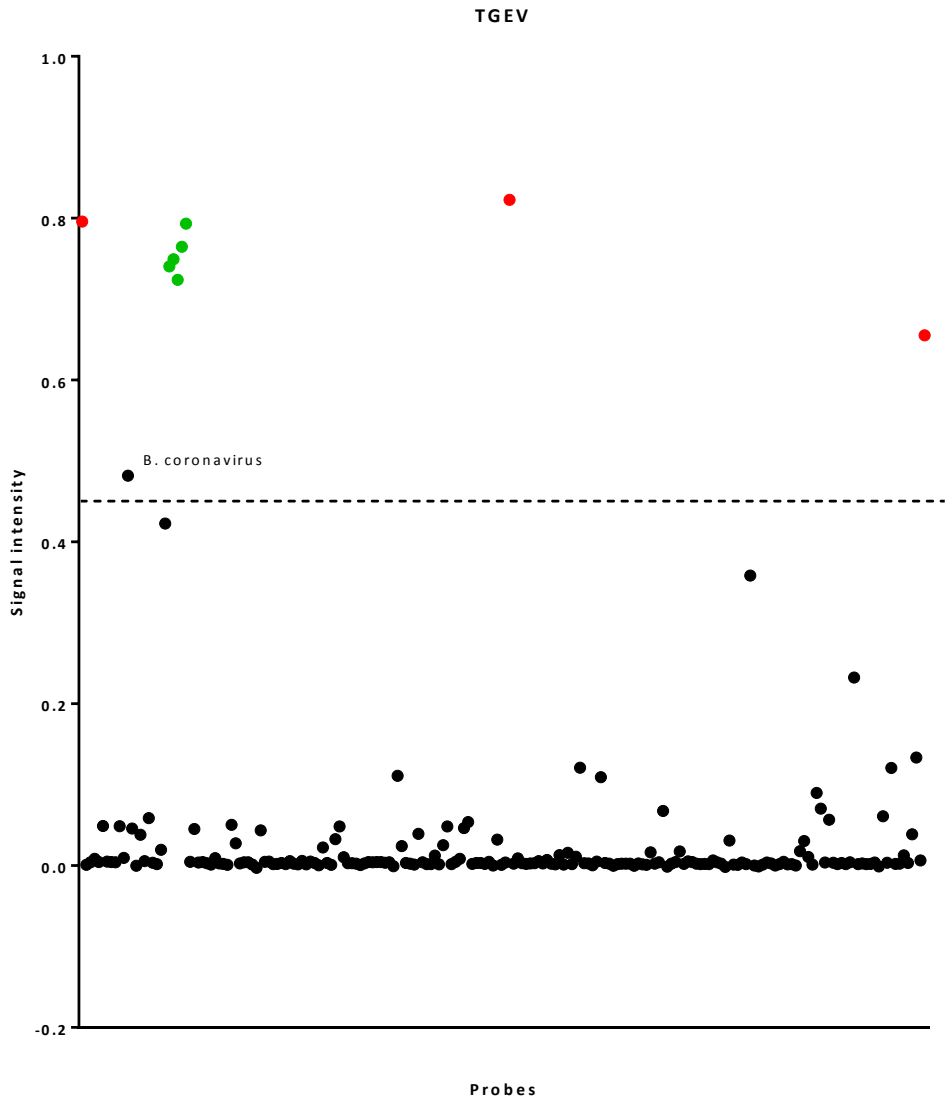


Figure 5.8.Normalised signal intensities of hybridised TGEV amplified by random amplification

Green spots correspond to TGEV probes – Red spots correspond to Biotin markers - Signal cut-off of 0.4 is represented by a dashed horizontal line.
Signal spot intensities of the 5 TGEV probes were also high varying from 0.72 to 0.79. One probe, corresponding to B. coronavirus showed positive hybridisation signals of 0.48. This probe represents a virus that belongs to the same family; nonetheless, its signal value remained slightly above the threshold signal intensity.

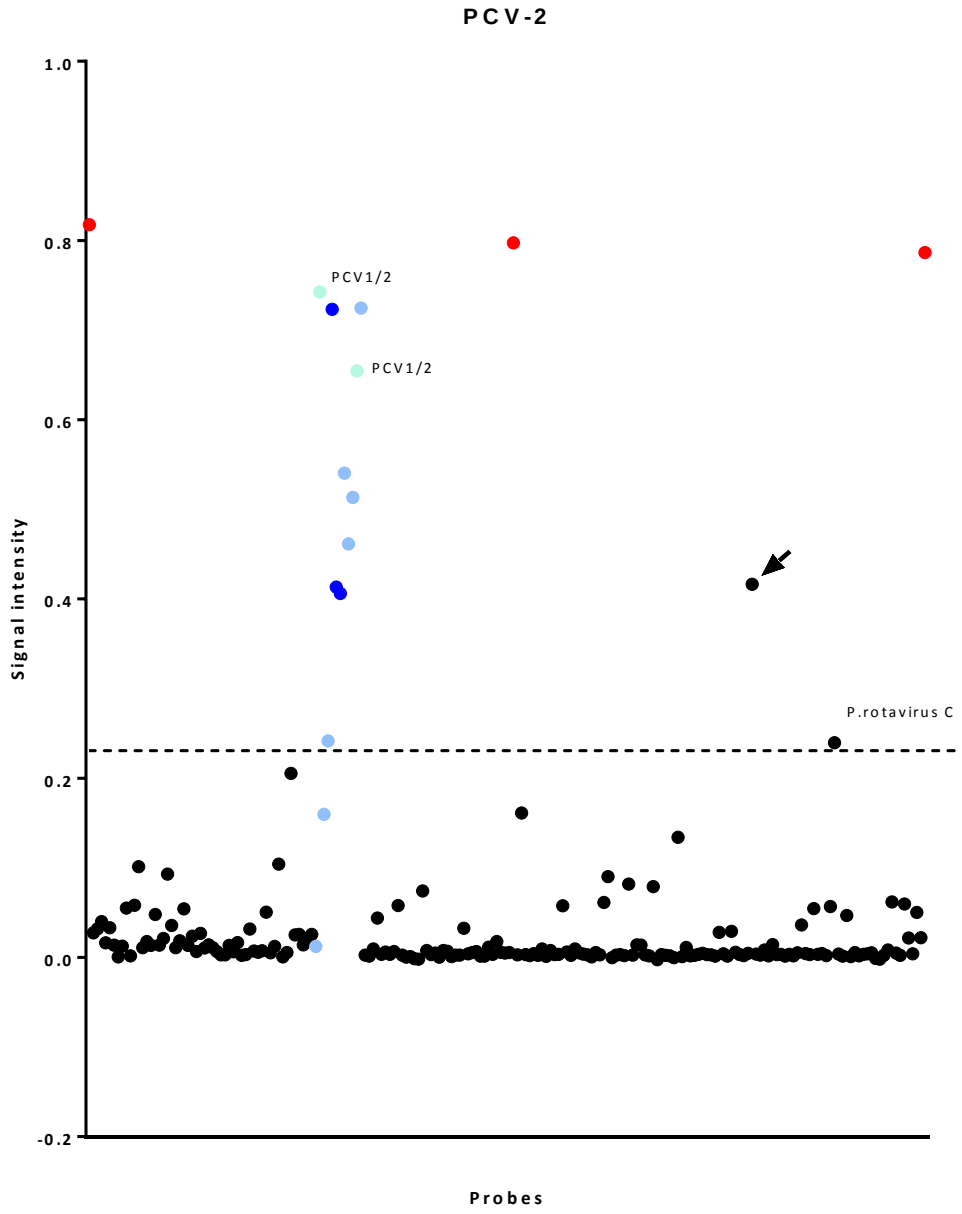


Figure 5.9. Normalised signal intensities of hybridised PCV-2 amplified by random amplification

Blue spots correspond to PCV-2 and light blue spots to PCV-1 probes – Red spots correspond to Biotin markers- Arrow indicates Por_rotA_3- Signal cut-off of 0.23 is represented by a dashed horizontal line. All PCV -2 probes had higher signal intensities than 0.23 and thus were regarded as positive. PCV1 probes were also present on the array with four PCV1 probes (Por_cir_0521, Por_cir_0523, Por_cir_0525, Por_cir_0529) (light blue spots) giving a positive signal. They were designed from the *rep* gene, a highly conserved region among porcine circoviruses. However, three other specific probes to PCV1 (Por_cir1_1, Por_cir1_3, and Por_cir1_4) exhibited lower signals than the threshold limit value. Por_cir1_2 and Por_cir_0527 (turquoise spots) have been also designed from the *rep* gene of a new type of PCV, which explains signal intensities of respectively 0.74 and 0.65.

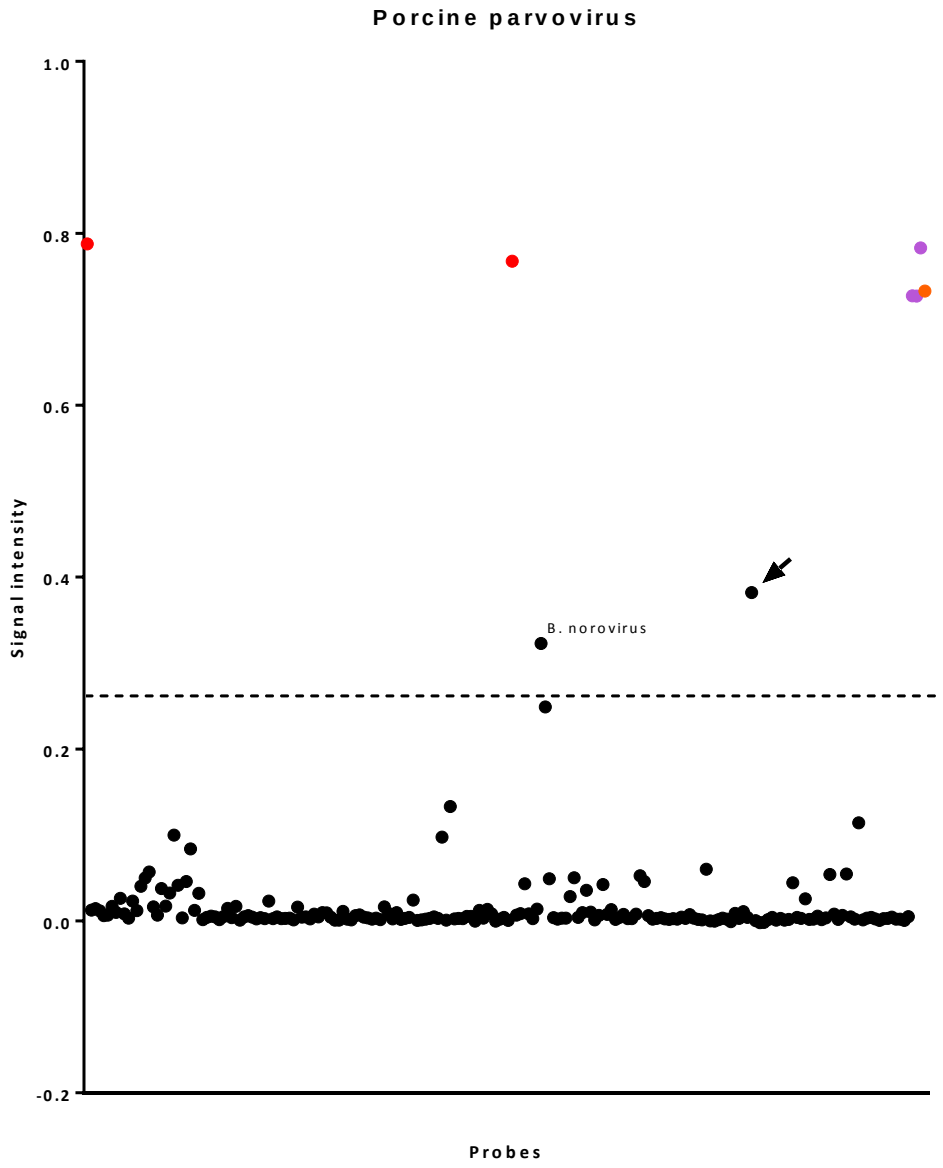


Figure 5.10. Normalised signal intensities of hybridised PPV amplified by random amplification

Purple spots correspond to PPV probes - Red spots correspond to Biotin markers - Signal cut-off of 0.26 is represented by a dashed horizontal line.
Only three probes representing porcine parvovirus in the array hybridised with signal intensities varying from 0.73 to 0.78. Only probe (B. norovirus) showed an intensity higher than the cut-off.

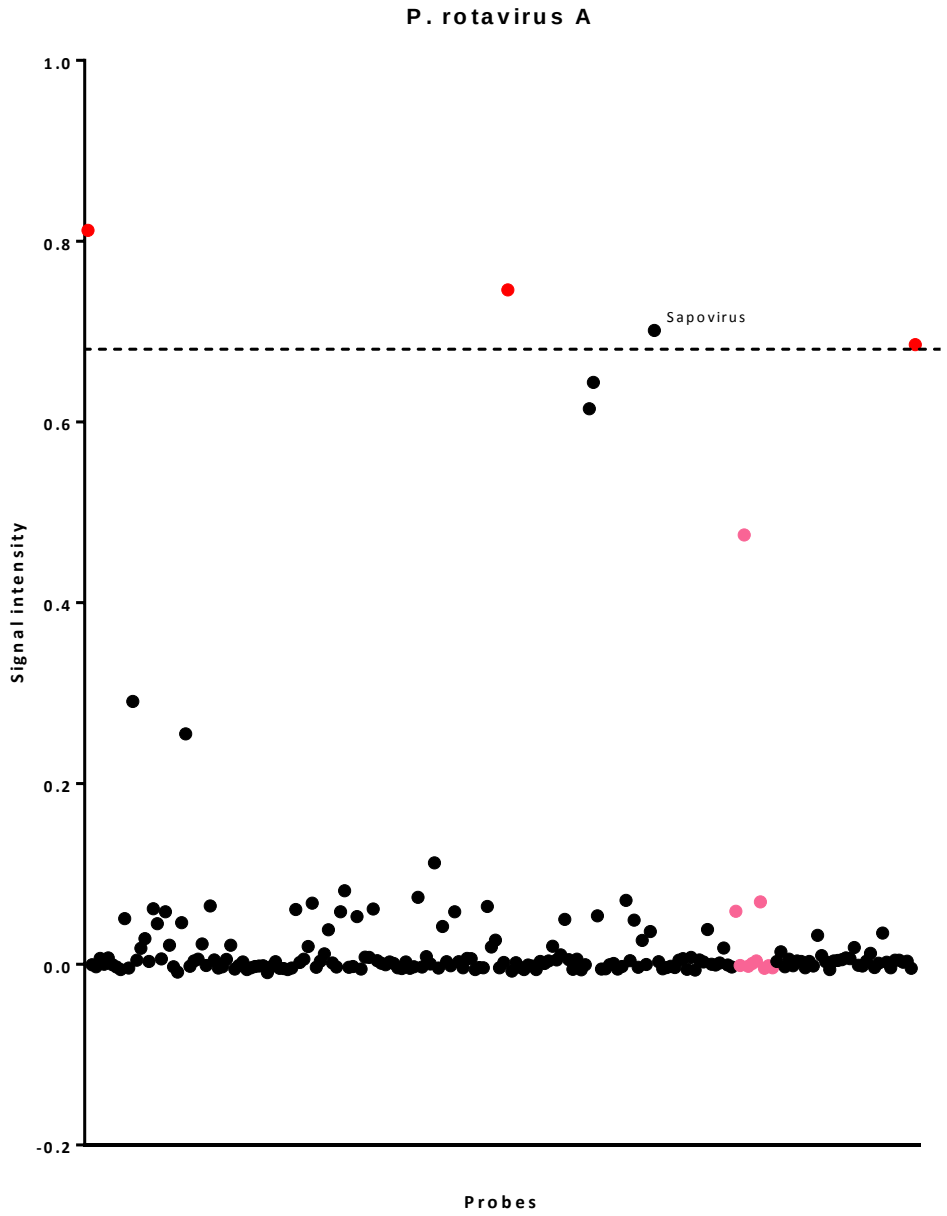


Figure 5.11. Normalised signal intensities of hybridised P. rotavius A amplified by random amplification

Pink spots correspond to P.rotavirus A probes – Red spots correspond to Biotin markers - Signal cut-off of 0.68 is represented by a dashed horizontal line.
Porcine rotavirus A is represented by 10 probes that showed no hybridisation, except from Por_rotA_3, with a signal intensity of 0.47, although below the cut-off. This particular probe was positive in almost all reference strains, PEDV, PCV2, PPV, and negative control. P. rotavirus A from this sample cross-hybridised with one sapovirus probe.

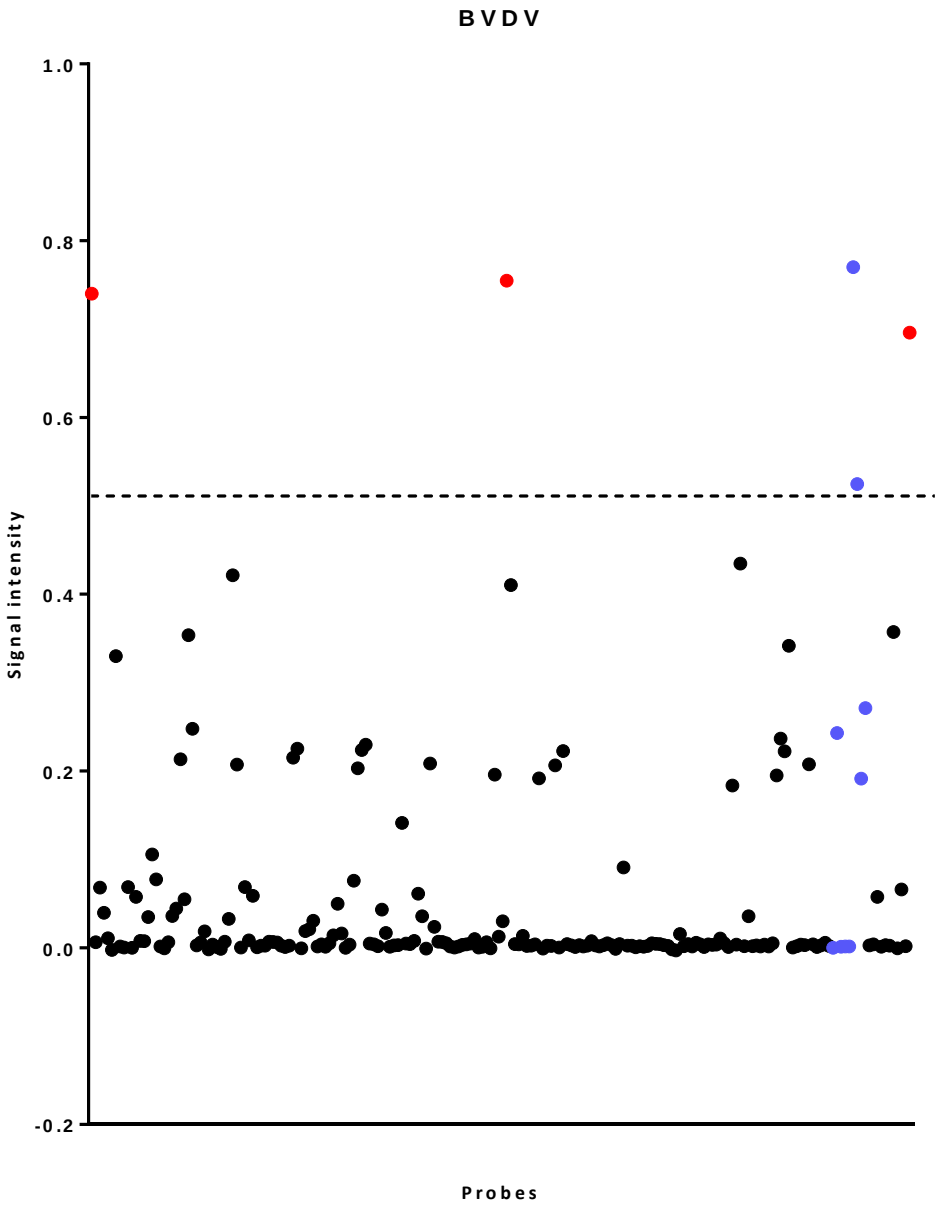


Figure 5.12. Normalised signal intensities of hybridised BVDV amplified by random amplification

Blue spots correspond to BVDV probes – Red spots correspond to Biotin markers. Signal cut-off of 0.51 is represented by a dashed horizontal line. For BVDV, two probes (BVDV_0160 and BVDV_0162) among nine showed high signal intensities of 0.77 and 0.52, respectively. Three probes, BVDV_0164, BVDV_0166 and BVDV2_2 (blue spots below the dashed line), are clearly distinguished from the basal signal intensity values.

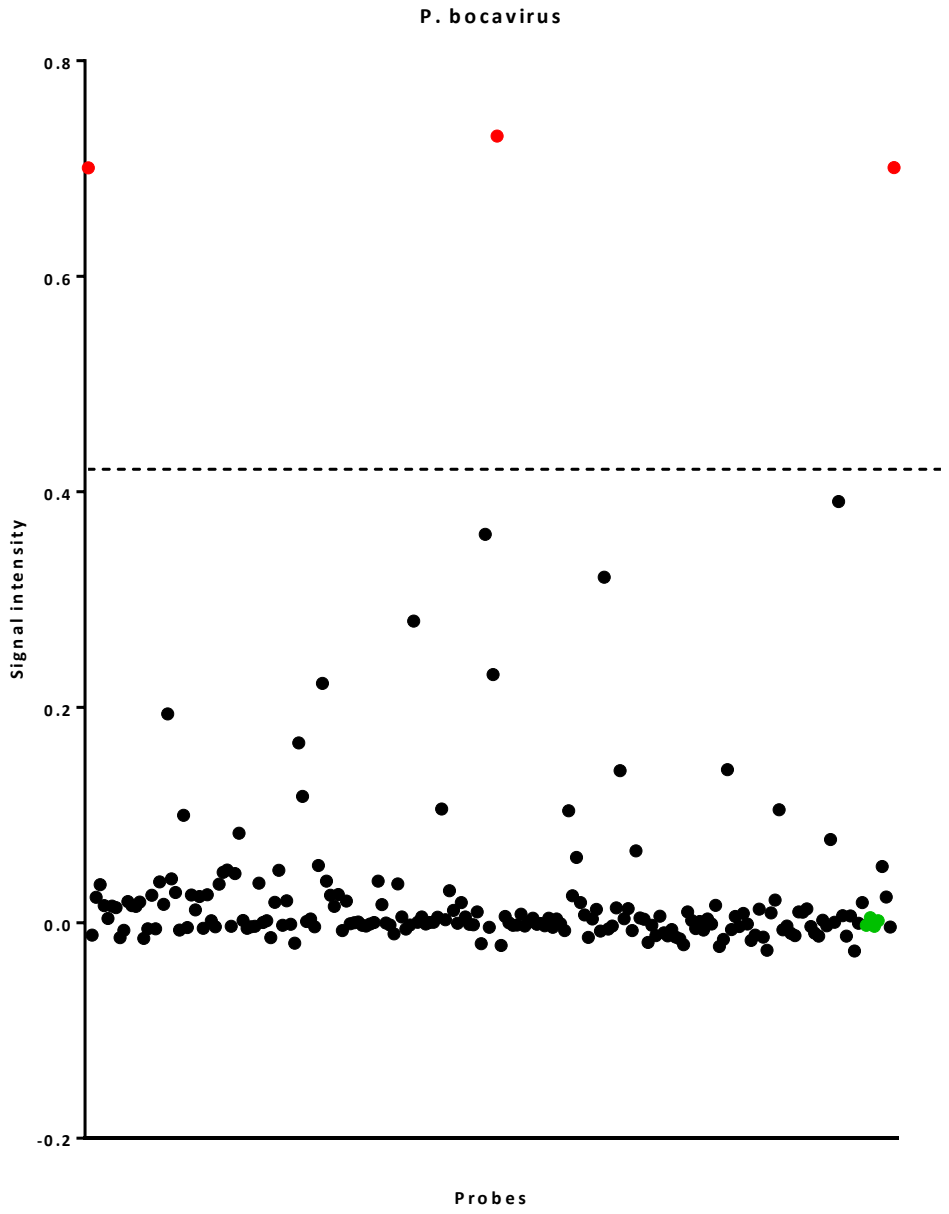


Figure 5.13. Normalised signal intensities of hybridised P. bocavirus amplified by random amplification

Green red spots correspond to P. bocavirus probes – Red spots correspond to Biotin markers - Signal cut-off of 0.42 is represented by a dashed horizontal line.
The four P. bocavirus probes did not hybridise with the P. bocavirus reference sample, with signal intensities fluctuating from -0.0023 to 0.0047.

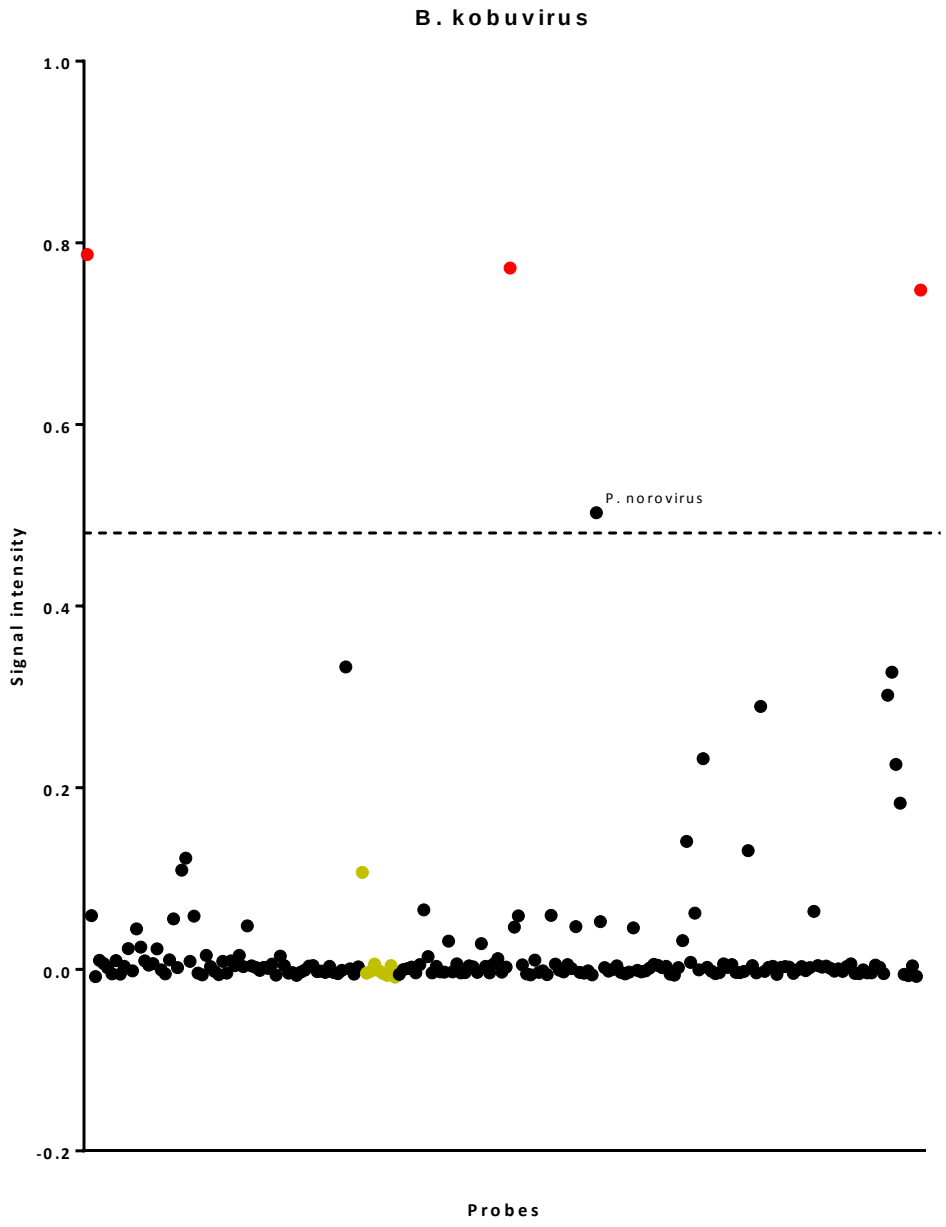


Figure 5.14. Normalised signal intensities of hybridised B. kobuvirus amplified by random amplification

Khaki spots correspond to B. kobuvirus probes – Red spots correspond to Biotin markers - Signal cut-off of 0.48 is represented by a dashed horizontal line.
All B. kobuvirus probes showed lower signal intensities to the detection limit value of 0.48, however the Porcine norovirus probe (P_nor_5) cross-hybridised with B. kobuvirus genome.

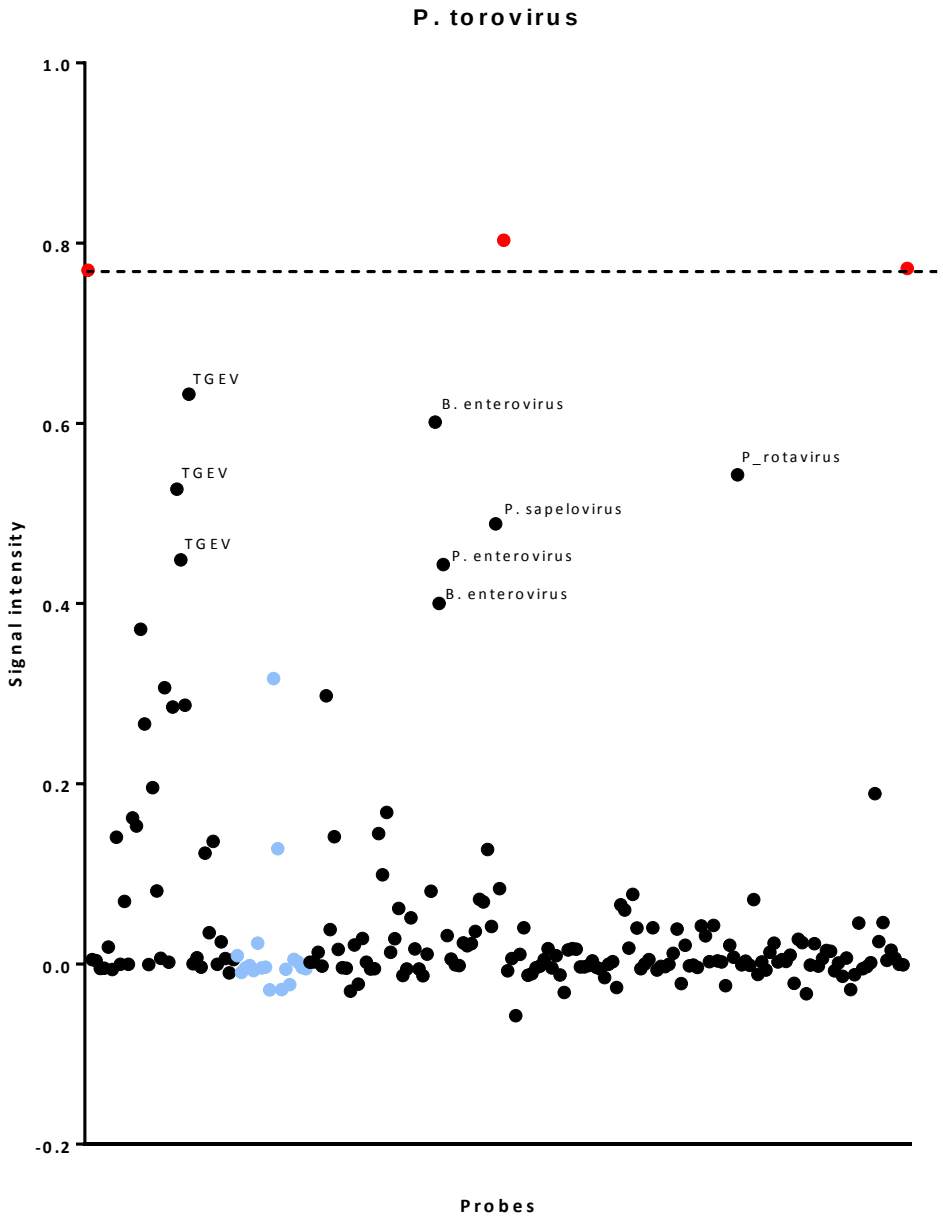


Figure 5.15. Normalised signal intensities of hybridised P. torovirus amplified by random amplification

Light blue spots correspond to P. torovirus probes – Red spots correspond to Biotin markers. Signal cut-off of 0.77 is represented by a dashed horizontal line. P. torovirus is represented by 18 probes designed from the nucleocapsid, membrane and hemagglutinin genes, which all showed negative hybridisation with the virus strain used. Three TGEV probes showed relatively high signal intensity, which was pedictible as they belong to the Coronaviridae family. Other Picornaviruses have also showed cross-reaction with the torovirus genome, although below the cut-off limit.

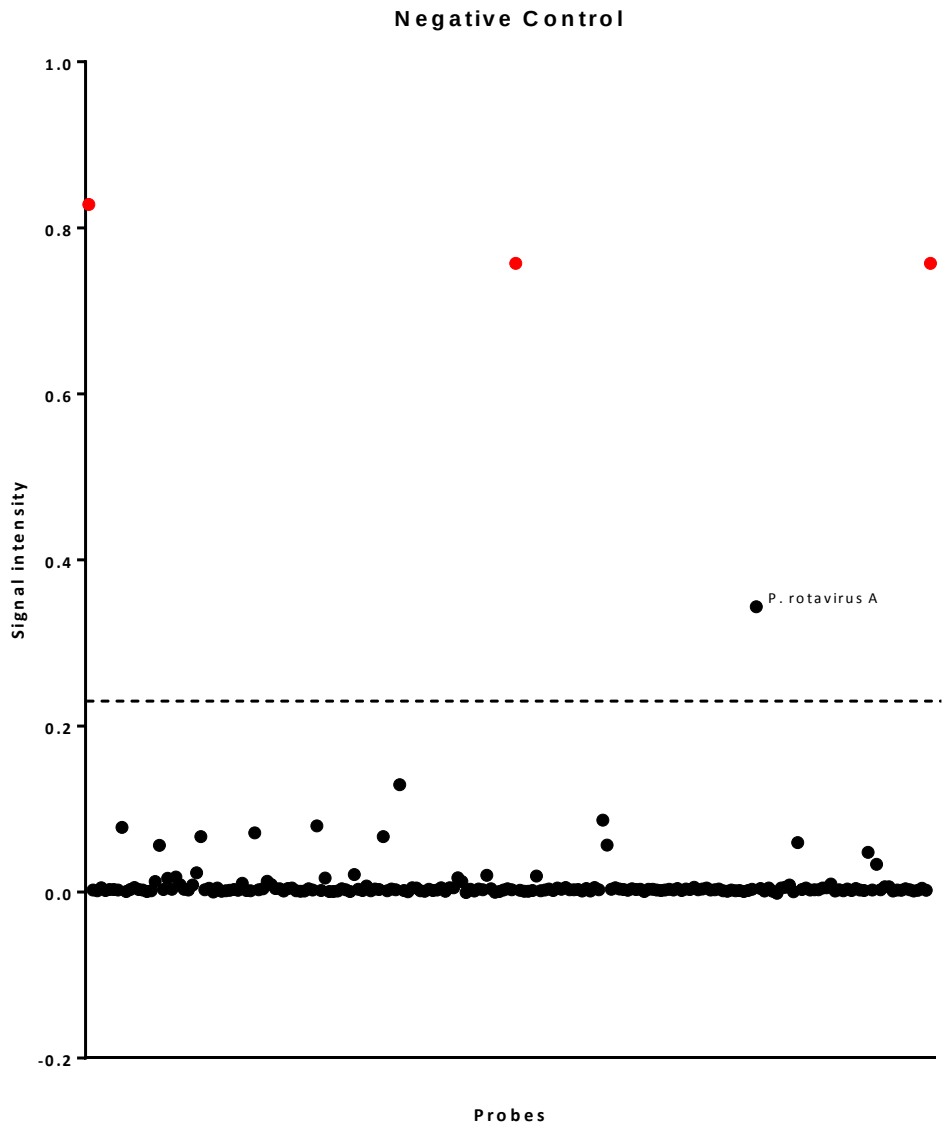


Figure 5.16. Normalised signal intensities of hybridised negative control amplified by random amplification

Nuclease free water was used as template - Red spots correspond to Biotin markers. Signal cut-off of 0.24 is represented by a dashed horizontal line. The negative control showed that all hybridised probes had signal intensities of around 0, the highest signal intensity below the detection limit, being 0.13, achieved by Bovine kobuvirus probe Bov_kob_0008. P. rotavirus A is positive in the negative control and many other virus hybridisation.

b. Array hybridisation with multiplexed reference strains

Two multiplex PCR reactions were performed with five reference strains each, comprising amplified genes from viruses, bacteria and parasites prepared by mixing single reference samples in equal proportions. The aim of this experiment was to test further the specificity of the array to discern the viruses in a mixture of strains. These consisted of two viruses (TGEV and PCV-2) and three bacteria (*S.* Typhimurium, *S.* Enteritidis and *C. difficile*) for Multiplex_1 and two bacteria (*B. pilosicoli* and *C. jejuni*), two viruses (PEDV and P. rotavirus A) and one parasite (*E. acervulina*) for Multiplex_2. The amplified PCR products were subjected to biotin labelling involving a random amplification step.

The array hybridisation with Multiplex_1 and Multiplex_2 are illustrated in figures 5.17 and 5.18 respectively.

In Multiplex_1, nearly all TGEV and PCV-2 probes correctly identified the corresponding viruses. The AT did not contain bacterial probes. Multiplex_2 hybridisation results on the array showed more cross hybridisation with target genes with less than half of the PEDV probes producing signals above the cut-off point, whereas the response from the P. rotavirus probes was extremely weak, equalling the background signal intensity.

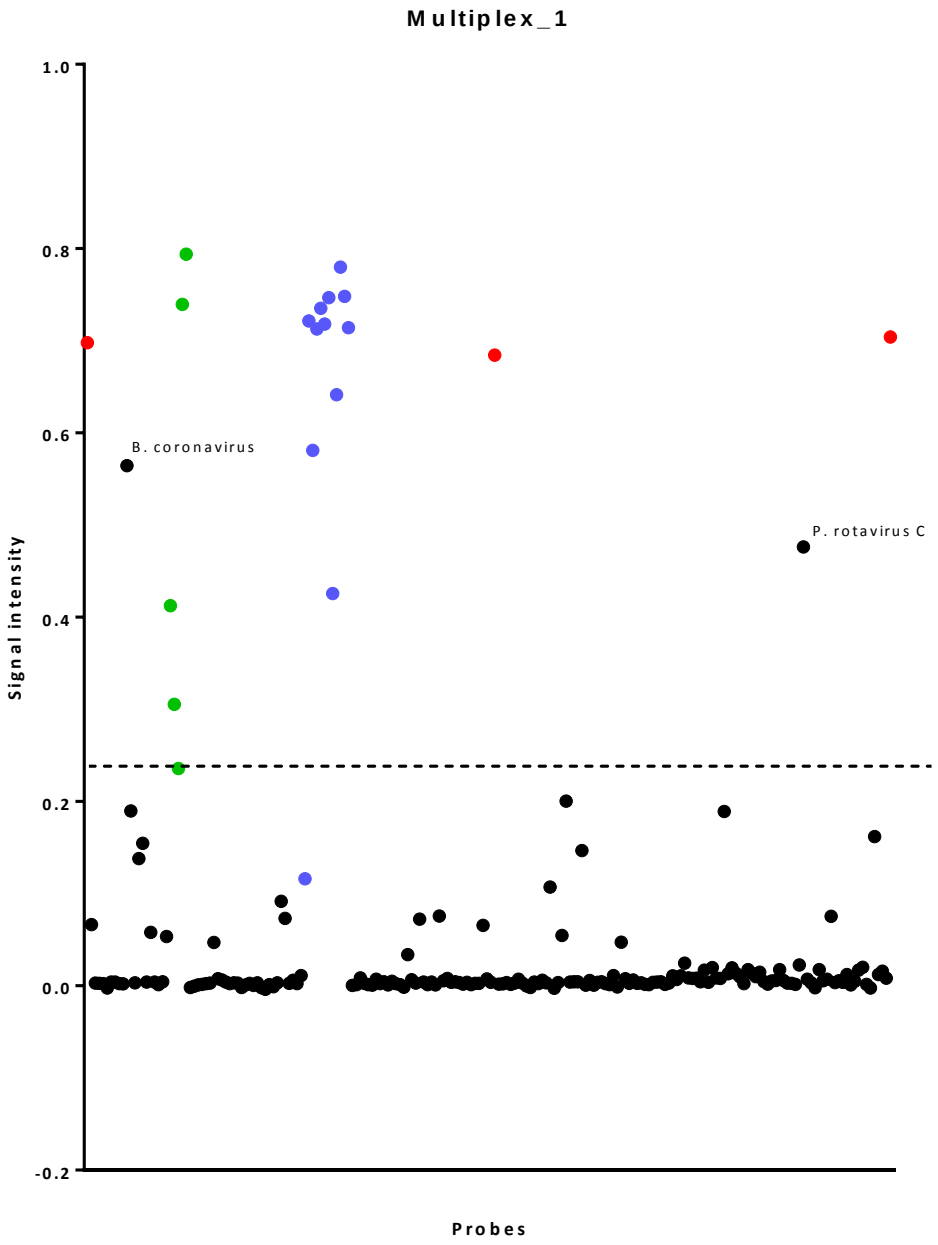


Figure 5.17. Normalised signal intensities of hybridised Multiplex_1. Hybridised with TGEV, PCV2, S. Typhimurium, *S.* Enteritidis and *C. difficile.*

Red spots correspond to Biotin markers, Green spots correspond to TGEV and blue spots correspond to PCV1/2. Signal cut-off of 0.24 is represented by a dashed horizontal line. Amplification was specific followed by random biotin labelling.
Nearly all PCV probes hybridised positively to their targets except from Por_cir1_1 that recorded a signal intensity of 0.12 (blue spot). One TGEV probe (green spot) was just at the limit of the cut-off signal. Two probes corresponding to B. coronavirus (B_cor_0229) and P. rotavirus C (P_rotC_8) cross-hybridised with the targets in the multiplex mixture of genes with respective signal intensities of 0.56 and 0.48.

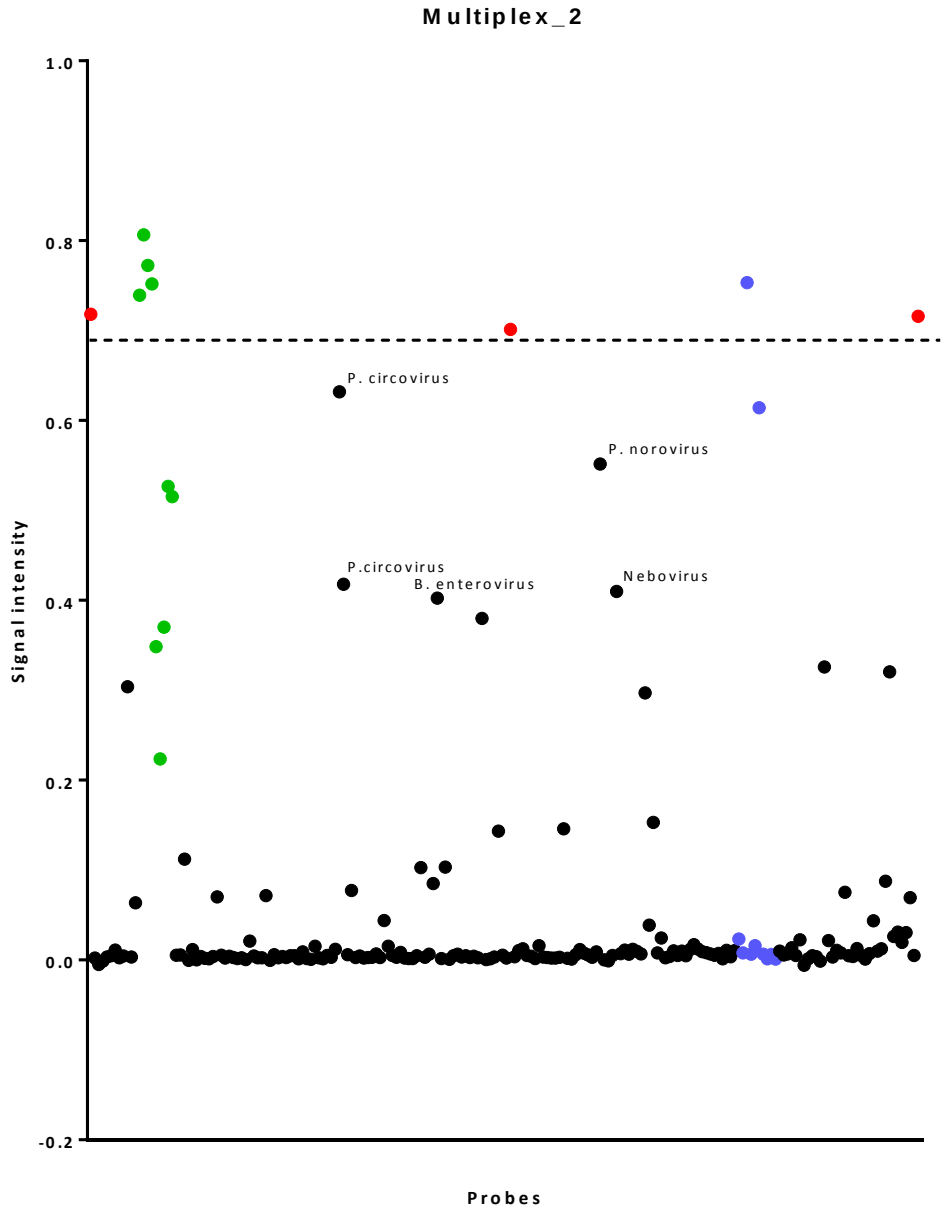


Figure 5.18. Normalised signal intensities of hybridised Multiplex_2. Hybridised with *B. pilosicoli*, PEDV, P. rotavirus A, *E. acervulina* and *C. jejuni*.

Red spots correspond to Biotin markers, Green spots correspond to PEDV and blue spots correspond to P. rotavirus A. Signal cut-off of 0.69 is represented by a dashed horizontal line. Amplification was specific followed by random biotin labelling.

PCV, P. norovirus, B. enterovirus and Nebovirus probes revealed relatively high intensities which contributed to a higher detection limit. Four of the nine PEDV probes were positive. Only one P. rotavirus A probe (P_rotA_3) was positive, however, high signals were recorded in nearly all performed hybridisation assays. The signal values of the eight remaining P. rotavirus probes being all around 0.

5. 3. 1. 2. Specificity of ArrayTube using non-targeted viruses

The specificity of the AT microarray was also assessed using three viruses that were not targeted by the array; Bunyamwera virus (B1), Schmallenberg virus (S) and Equine influenza A virus (H3N8), and are shown in figures 5.19, 5.20 and 5.21 respectively.

Hybridisation of Schmallenberg and Bunyamwera viruses on the AT showed a high level of cross-hybridisation. Eighteen probes showed high signal intensities when hybridised with Schmallenberg virus and 12 probes with Bunyamwera virus. Eleven probes hybridised with both viruses. However, these probes intensities remain below the cut-off limit. Less hybridisation noise was noted when Equine influenza A virus was hybridised to the array. Por_rotA_3 probe hybridised with all of the three viruses, with a signal of 0.83 for Schmallemberg and Bunyamwera virus and 0.42 for Equine influenza virus.

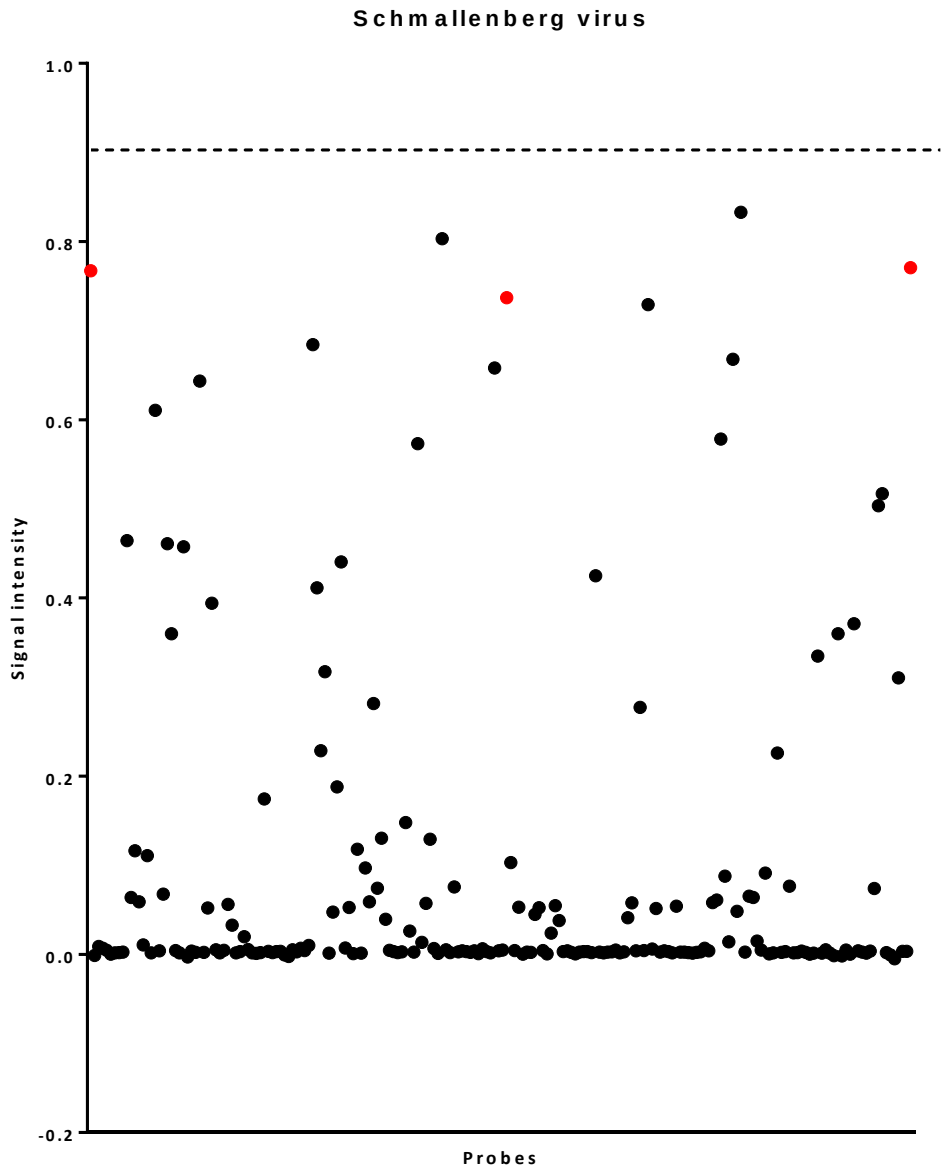


Figure 5.19. Normalised signal intensities of hybridised Schmallenberg virus

Red spots correspond to Biotin markers. Signal cut-0.91 is represented by a dashed horizontal line. Amplification was random and hybridisation indicated a high background consisting of non specific cross-reaction with probes corresponding to other viruses. This has led to high threshold, higher than the biotin markers signal intensity.

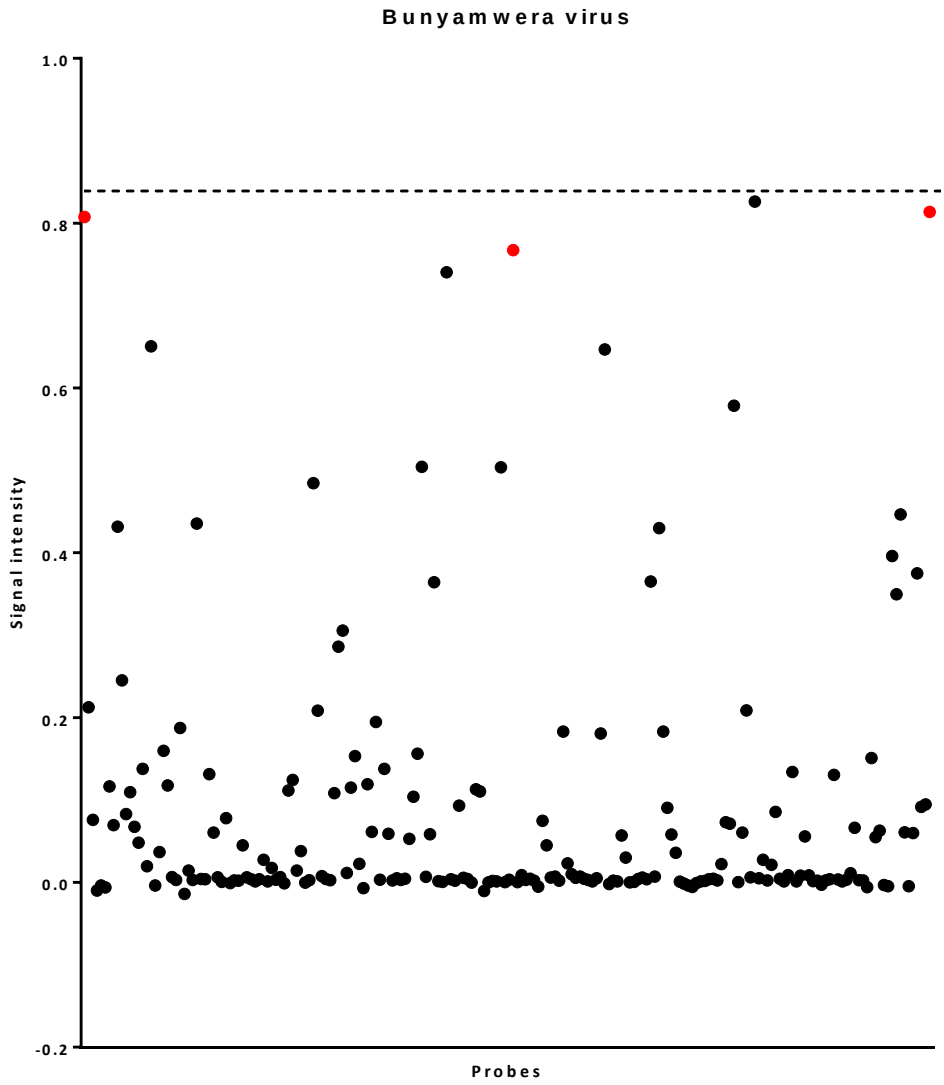


Figure 5.20. Normalised signal intensities of hybridised Bunyamwera virus

Red spots correspond to Biotin markers. Signal cut-off of 0.84 is represented by a dashed horizontal line. Amplification was random and hybridisation to the array showed that Bunyamwera virus genome viruses cross-hybridised with the probes on the array. The detection limit was also set to a signal value higher than the biotin markers signal intensity.

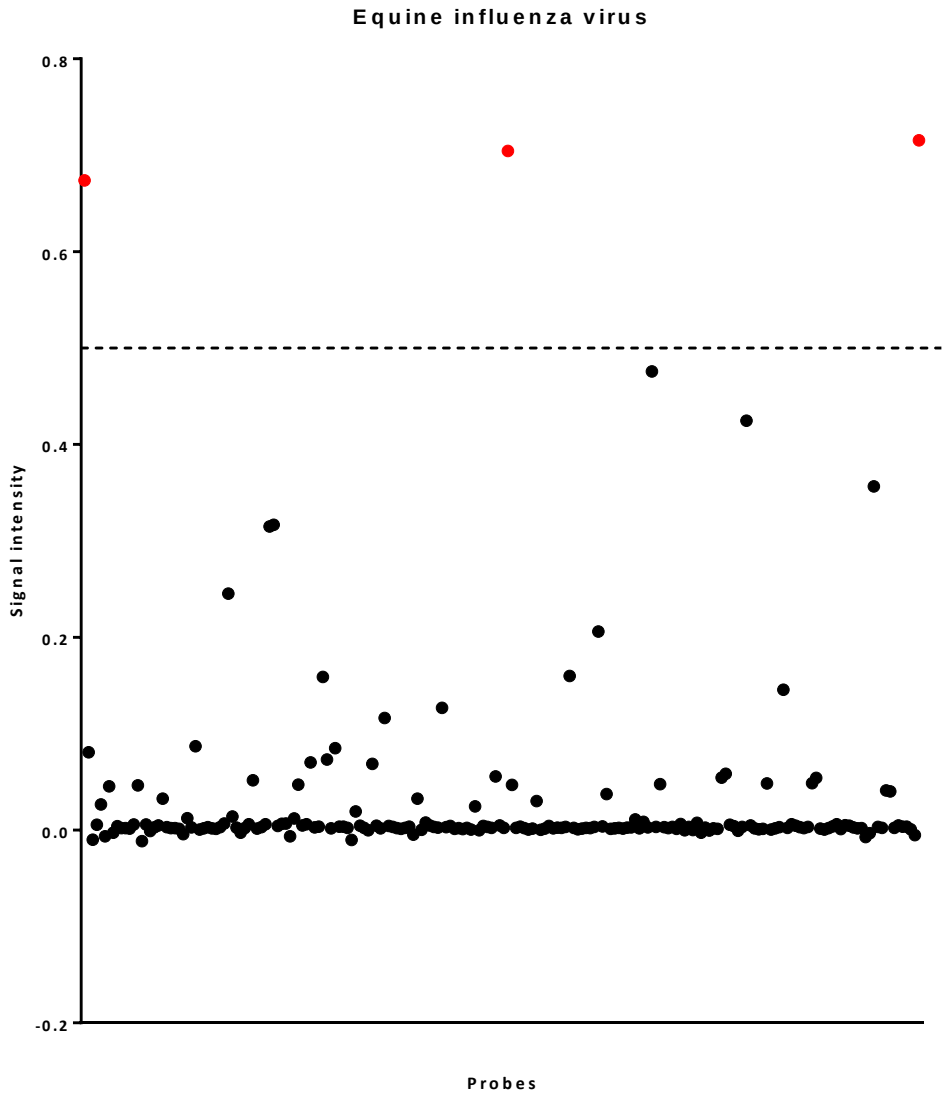


Figure 5.21. Normalised signal intensities of hybridised Equine influenza virus

Red spots correspond to Biotin markers. Signal cut-off of 0.50 is represented by a dashed horizontal line. Amplification was random, hybridisation showed no cross-hybridising probes.

5. 3. 1. 3. Comparison of hybridisation after separate random and specific amplification

PCV-2 and TGEV were subjected to two different strategies of amplification prior to biotin-labelling. PCR products from both reactions were obtained using random primer A, primer B (primer A tag) and primers specific for PCV-2 and TGEV respectively with random and specific amplification. They were then labelled with biotin and hybridised individually on ATs.

The hybridisation results of random and specific amplification of TGEV (Fig. 5.22) and PCV-2 (Fig.5.23), showed that hybridisation after random amplification performed better than after specific amplification. Signal intensities were clearly higher in the specific compared to the random amplification and there were fewer non-specific probes giving signals approaching or exceeding the cut-off. For TGEV, 5 out of 5 probes were positive in the random amplification, whereas only 2 out of 5 were positive in the specific amplification. As for PCV, the random amplification led to 10 out of 12 probes to generate signal intensities above the cut-off, whereas 5 out of 12 probes hybridised to PCV probes in the specific amplification.

In both PCV-2 and TGEV hybridisation results of specific amplification reactions, a high background noise was noticeable, also a higher number of probes cross-hybridised with the viruses. However signal intensities of positive probes were relatively higher compared to the random.

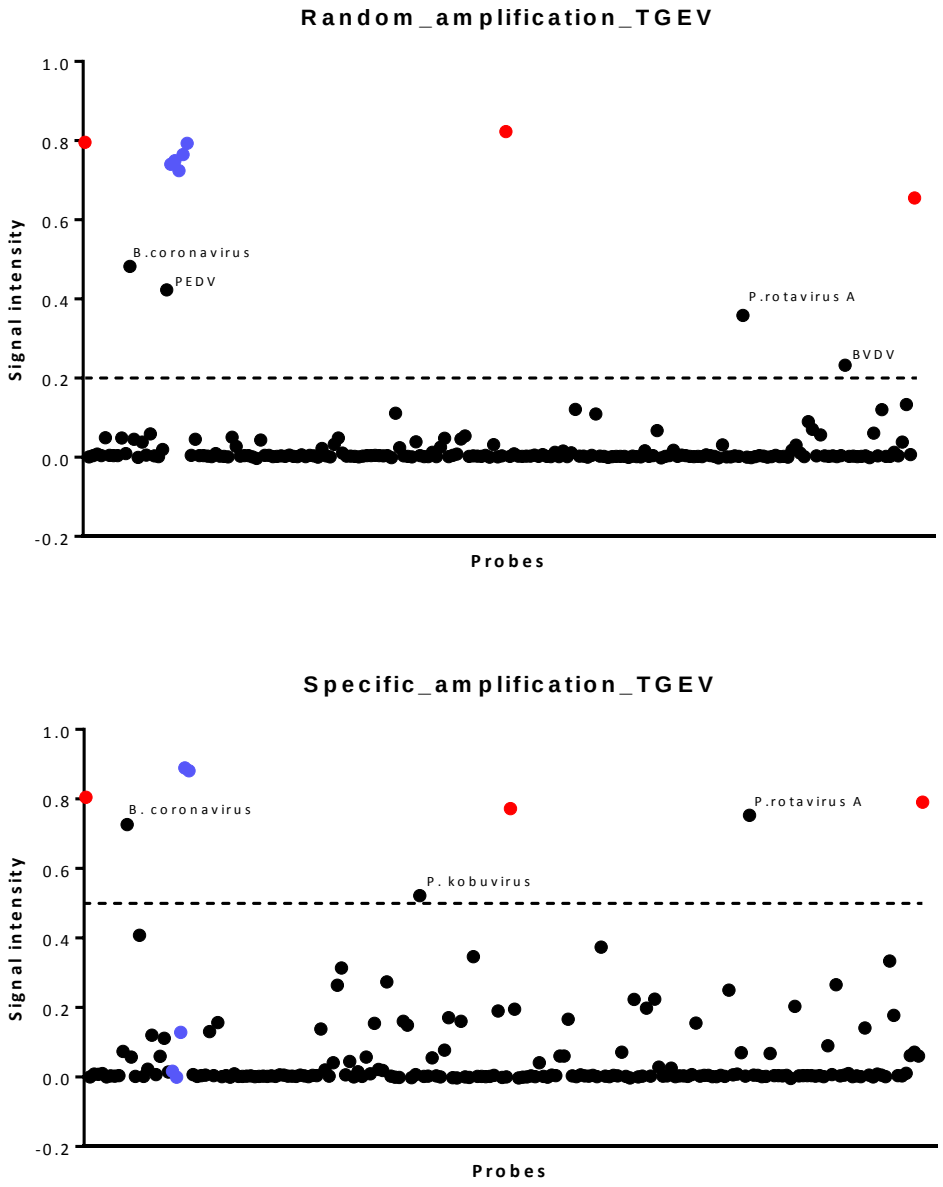


Figure 5.22. Hybridisation after Random and specific amplification of TGEV

Red spots correspond to Biotin markers and Blue spot to TGEV. Signal cut-off of 0.20 and 0.50 for the random and the specific assays are represented by a dashed horizontal line. Five representative probes of TGEV were positive with intensities varying between 0.72 and 0.79 in the random amplification compared to two probes in the specific amplification. However, signal intensities of the positive probes in the specific amplification (0.88 and 0.89) were visibly higher, exceeding the biotin markers signal. All coronaviruses probes intensities (Coronavirus, PEDV and Toroviruses) were not included in the calculation of the cut-off, as they belong to the same family than TGEV and might have shared gene sequences .

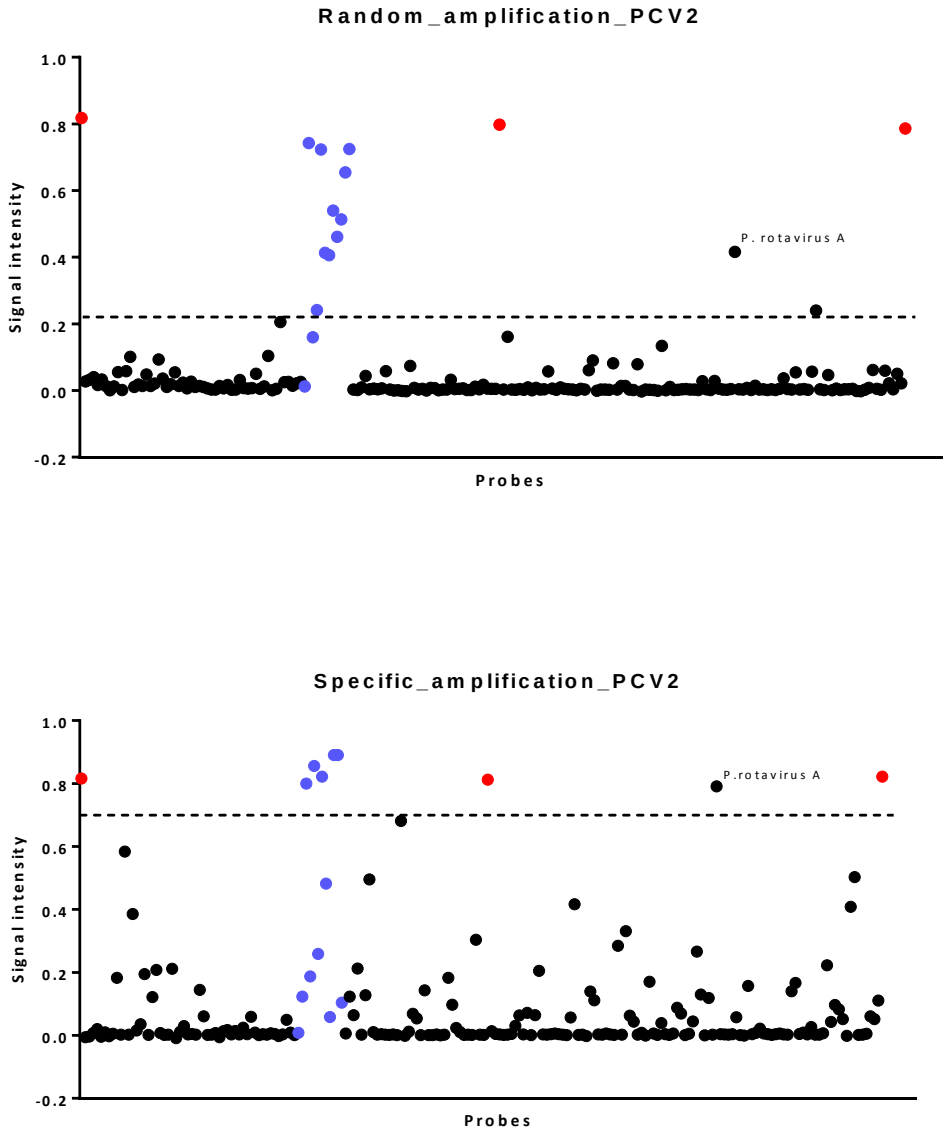


Figure 5.23. Hybridisation after Random and specific amplification of PCV-2

Red spots correspond to Biotin markers and Blue spot to PCV. Signal cut-off of 0.22 and 0.70 for the random and the specific amplification are represented by a dashed horizontal line. All porcine circoviruses probes represent PCV-1 and PCV-2. Hybridisation following random amplification showed that ten out of 12 probes were positive compared to five positive out of 12 in the specific amplification. The major difference between the two hybridisation settings is that signal intensities are markedly higher in the specific amplification, with a lowest value of 0.48 and highest value of 0.89. However in the random reaction, more representative probes were positive.

5. 3. 1. 4. Hybridisation with host genomic DNA

To assess cross-hybridisation of genomic host DNA with probes on the array, PCR products of bovine and porcine cytochrome b were randomly amplified and biotin labelled and subsequently hybridised to the AT.

Cytochrome b genes were amplified from porcine and bovine host DNA, Fig. 5.24 shows two distinct bands of 1074 bp for the bovine cytochrome b PCR product and 1080 bp for porcine cytochrome b.

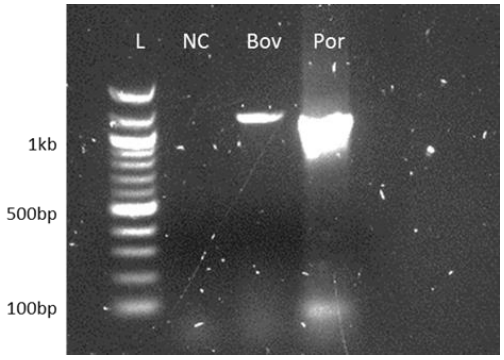


Figure 5.24. Agarose gel electrophoresis of specific amplification of Cytochrome b.

L: 100 bp DNA ladder, NC: Negative control, Bov: Bovine cytochrome B, Por: Porcine cytochrome b – Bovine cytochrome b from bovine and porcine hosts were amplified with amplicon size of 1074 bp and 1080 bp respectively as shown in lane Bov and Por.

Array hybridisation of bovine cytochrome b (Fig. 5.25) showed a low background signal level. Only one probe of PCV hybridised to the bovine genome but with a low signal intensity below the cut-off limit. However, the signal was sufficient to generate a faded spot in the array. Porcine cytochrome hybridisation to the AT showed a relatively different pattern compared to the configuration of the bovine cytochrome b hybridisation (Fig. 5.26), with background noise characterised by relatively high signal. Ultimately, probe Por_rotA_3 (labelled in both figures) also showed a positive hybridisation.

However, this cannot be attributed to cross-hybridisation as it was positive in all ATs.

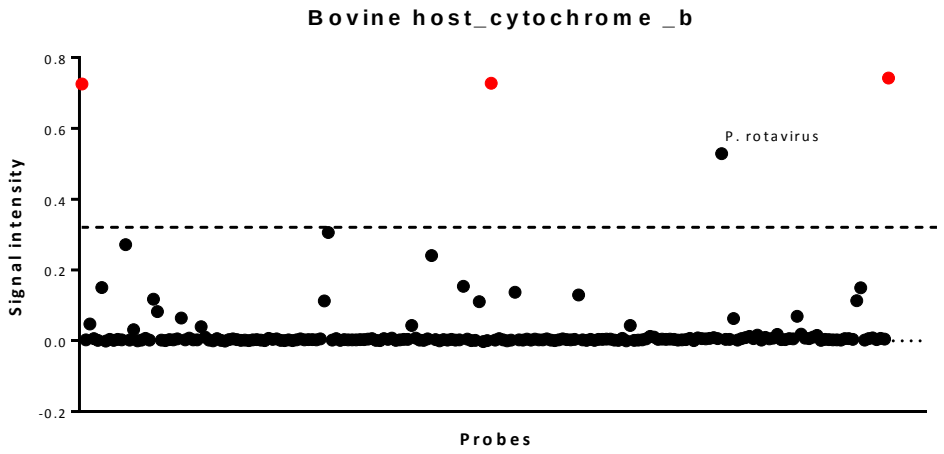


Figure 5.25. Normalised signal intensities of hybridised bovine host cytochrome b

Red spots correspond to Biotin markers. Signal cut-off of 0.32 is represented by a dashed horizontal line. The highest signal intensity recorded corresponds to Por_cir_0521 (0.30) which is just below the cut-off line. This signal intensity is below the limit of detection.

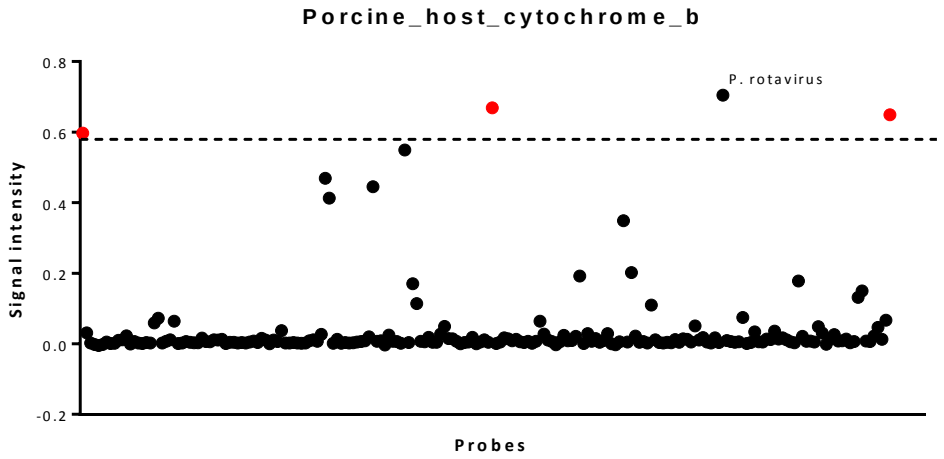


Figure 5.26. Normalised signal intensities of hybridised porcine host cytochrome b

Red spots correspond to Biotin markers. Signal cut-off of 0.58 is represented by a dashed horizontal line. Four probes had moderately higher signals. Por_cir2_3 with a signal intensity of 0.47, Por_cir_0521 with 0.41, Bov_kob_0006 with 0.44 and Por_kob_6 with 0.55. The spots on the array were darker for circovirus probes, but invisible for kobuvirus probes despite the highest signal intensity that was reached by Por_kob_6 probe.

5. 3. 2. Sensitivity of the ArrayTube platform

Two-fold serial dilutions of PCV-2 DNA and TGEV RNA, were used in a specific PCR to detect the viruses. The amplicons were randomly amplified and labelled with biotin and then hybridised to the AT.

a. PCV-2

Figure 5.27, shows that the PCR reactions of diluted PCV2 DNA produced the expected amplicon of 543 bp for the first four dilutions with their respective copy numbers. The thickness of the bands is clearly diminishing along with the dilutions. No bands were visible in lanes E to H with lower copy numbers although surprisingly this was still 2.1 x 10^9 copies (2 ng).

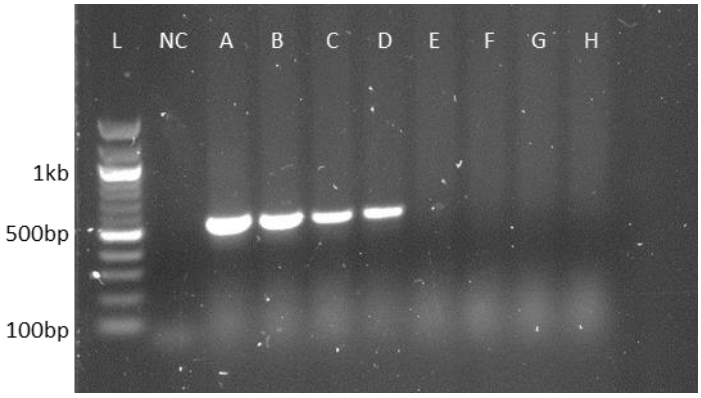


Figure 5.27. Agarose gel electrophoresis of PCV-2 (Capsid - replicase) amplification in two-fold serial dilution of DNA.

Amplicon size: 543 bp. L: 100 bp DNA ladder, NC: Negative control, A: 2.065 x 10^{10}; B: 1.033 x 10^{10}, C: 5.164 x 10^9, D: 2.065 x 10^9, E: 1.033 x 10^9, F: 5.164 x 10^8, G: 2.582 x 10^8, H: 1.291 x 10^8. DNA concentrations are 20 ng, 10 ng, 5 ng, 2 ng, 1 ng, 0.5 ng, 0.25 ng and 0.125 ng per µl, corresponding respectively to the copy number A to H.

Hybridisations of labelled PCR products are illustrated in Figs. 5.28 – 5.35 (below). The twelve probes on the array represent both PCV genotype 1 and 2 (blue spots), among them, eight probes showed a positive hybridisation with a signal intensity higher than 0.61 for the sample containing 2.065×10^{10} copies of PCV2 genome (20 ng). Similarly, eight probes were able to hybridise with a lower copy number (1.033×10^{10}). For the dilutions 5 ng and 2 ng, corresponding to 5.164×10^{9} and 2.065×10^{9} copies, respectively, only five probes hybridised to the targets, albeit with high signal intensities. For higher dilutions (copy numbers: 1.033×10^{9}, 5.164×10^{8}, 2.582×10^{8} and 1.291×10^{8}, equivalent to 1 ng – 0.125 ng), no probe showed a signal with intensity higher than the cut-off. It should be noted that PCV targets cross-reacted with one of the probes of Porcine kobuvirus and Bovine torovirus.

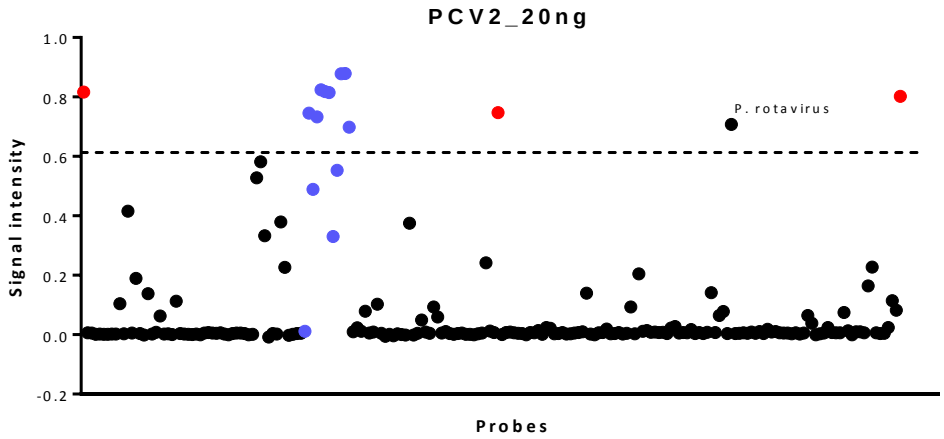


Figure 5.28. Normalised signal intensities of hybridised 20ng / µl (2.065 x 10^{10} copies number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.61 is represented by a dashed horizontal line. Eight probes among 12 showed a higher signal intensity above the cut-off value.

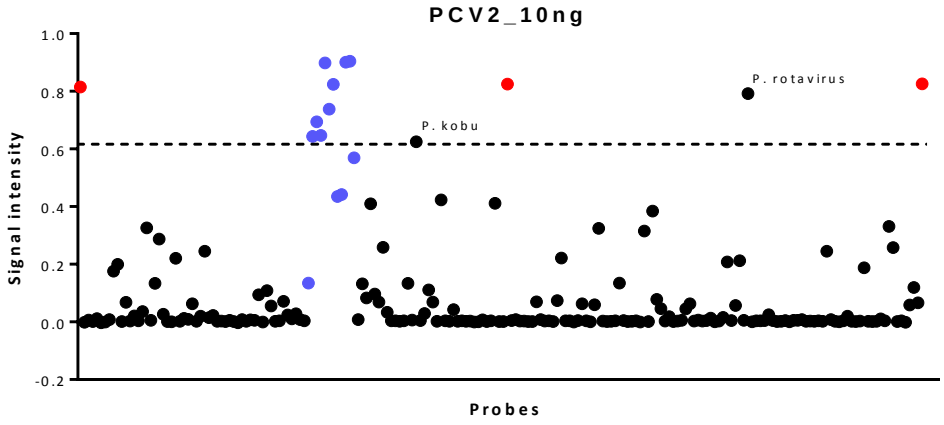


Figure 5.29. Normalised signal intensities of hybridised 10ng / µl (1.033 x 10^{10} copy number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.61 is represented by a dashed horizontal line. Eight probes showed a lower signal intensity than the threshold of 0.61.

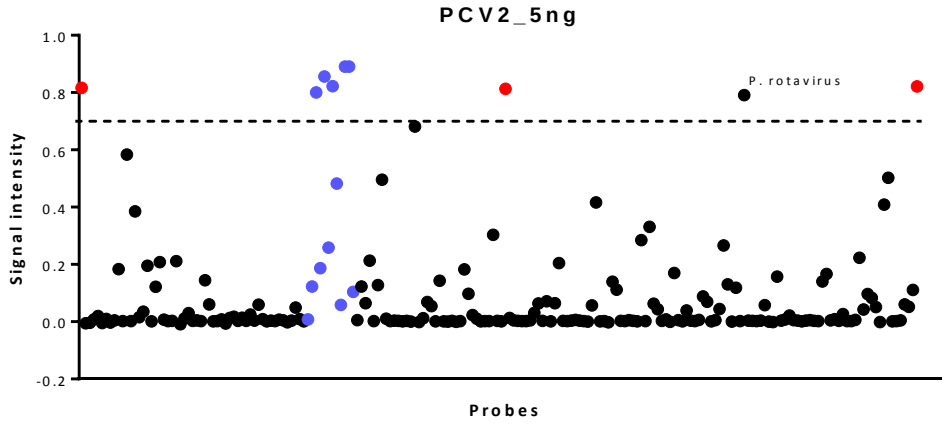


Figure 5.30. Normalised signal intensities of hybridised 5 ng / µl (5.164 x 10^9 copy number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.70 is represented by a dashed horizontal line. Only 50% of PCV probes were able to hybridise to the array.

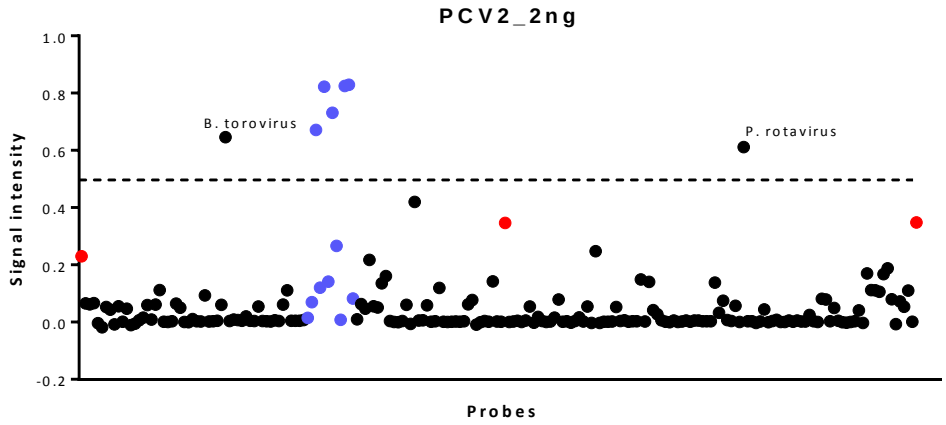


Figure 5.31. Normalised signal intensities of hybridised 2 ng / µl (2.065 x 10^9 copy number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.49 is represented by a dashed horizontal line. Five probes out of 12 were positive with signal intensity varying between 0.67 and 0.83. In this assay, biotin markers showed unusually low signal intensity.

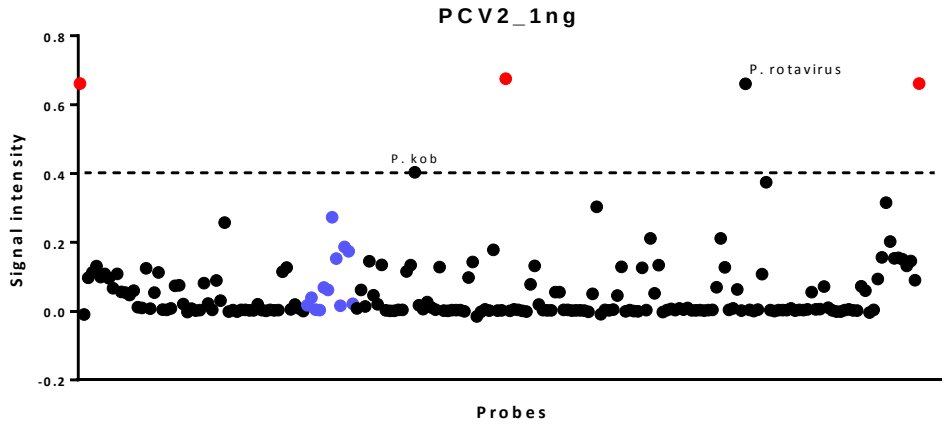


Figure 5.32. Normalised signal intensities of hybridised 1 ng / µl (1.033 x 10^9 copy number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.4 is represented by a dashed horizontal line. No hybridisation of PCV probes was recorded.

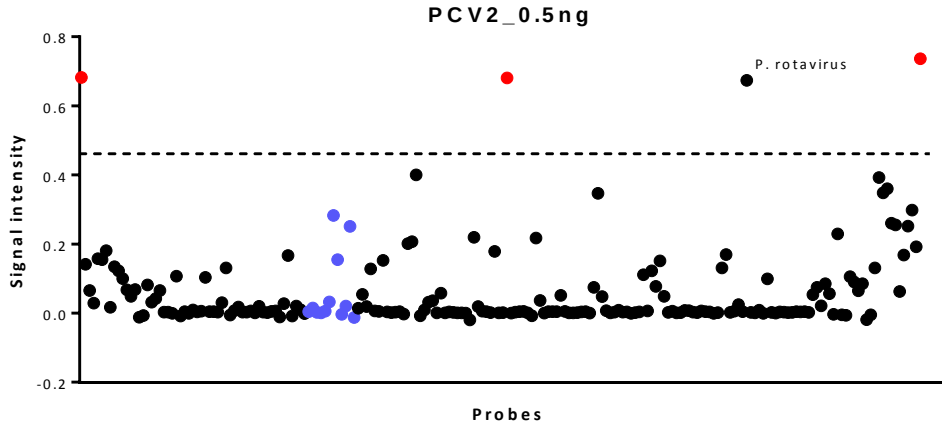


Figure 5.33. Normalised signal intensities of hybridised 0.5 ng / µl (5.164 x 10^8 copy number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.46 is represented by a dashed horizontal line. All PCV probes had lower intensity than the signal cutoff.

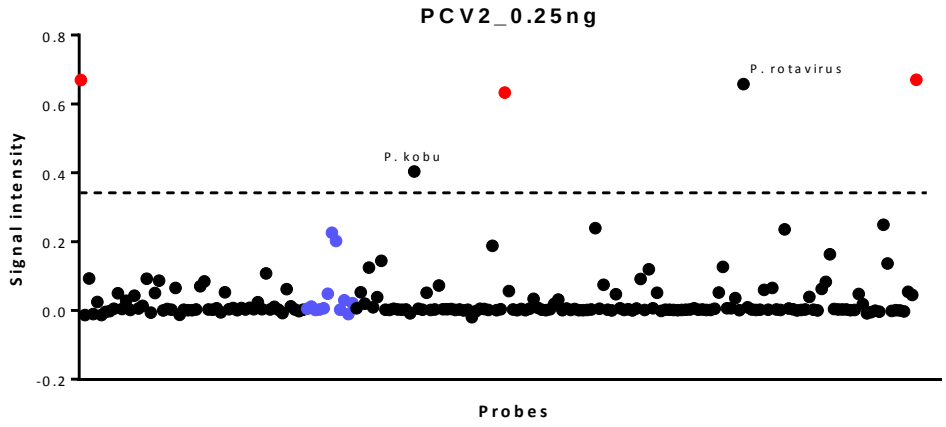


Figure 5.34. Normalised signal intensities of hybridised 0.25 ng / µl (2.582 x 10^8 copy number) PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. . Signal cut-off of 0.34 is represented by a dashed horizontal line. No probe produced a signal with intensity higher than 0.34, except from Por_kob_6 (indicated in the figure).

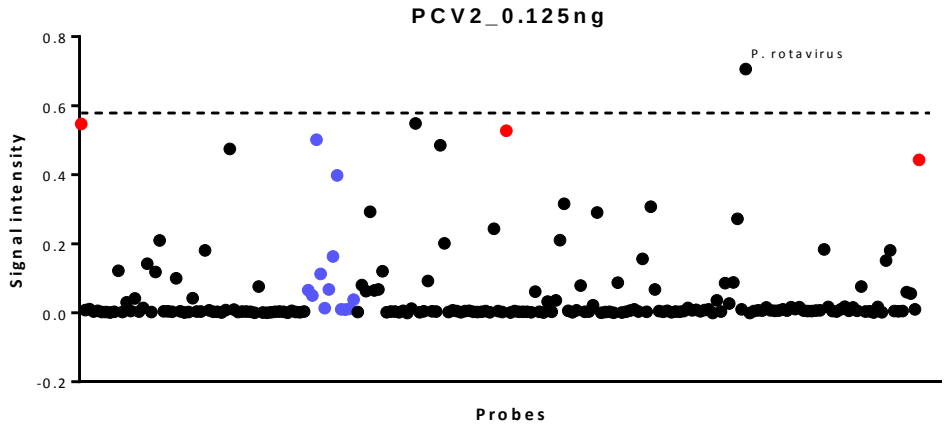


Figure 5.35. Normalised signal intensities of hybridised 0.125 ng/µl (1.291 x 10^8 copy number) of PCV2

Red spots correspond to Biotin markers; Blue spots correspond to P. circovirus. Signal cut-off of 0.58 is represented by a dashed horizontal line. The cut-off limit was high as numerous probes contributed to high background noise, the biotin markers intensity were relatively low compared to the usual 0.70-0.80.

b. TGEV

TGEV RNA was serially diluted in 2-fold steps, reverse transcribed and a fragment of 1388 bp was amplified with specific primers for all the dilutions. The electrophoresis gel image (Fig. 5.36) shows the expected amplicon size for all dilutions with lane A being the non-diluted template (61.61ng). The brightness fell with the dilutions and appeared to be proportional to the cDNA concentration in the sample. The viral gene was detectable until the template concentration reached 0.015ng, corresponding to 9.0308 x 10^5 copies (lane L). A faint band was observable in lane M (0.0078ng – 4.84 x 10^5). However, non-specific amplification was also noticeable in the same lane. The same non-specific PCR product is present in lane N.

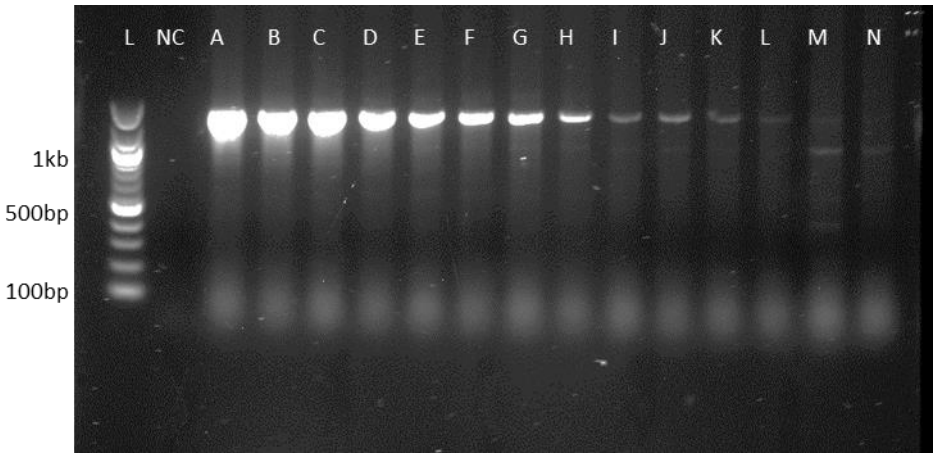


Figure 5.36. Agarose gel electrophoresis of TGEV (ORF1b) amplification in two-fold serial dilution of RNA

Amplicon size: 1388 bp. L: 100bp DNA ladder, NC: Negative control, A PCR product of 1388 bp was obtained after specific amplification. The 2- fold step diluted amount of cDNA in the PCR reaction varied from 20ng/µl to 0.0078ng/µl, corresponding to lanes A to N. Copy number were by lane A: 3.823 x 10^9, B: 1.241 x 10^9, C: 6.205 x 10^8, D: 3.103 x 10^8, E: 1.241 x 10^8, F: 6.205 x 10^7, G: 3.103 x 10^7, H: 1.551 x 10^7, I: 7.756 x 10^6, J: 3.847 x 10^6, K: 1.924 x 10^6, L: 9.308 x 10^5, M: 4.840 x 10^5, N: 2.420 x 10^5.

The hybridisation on the AT of biotinylated TGEV PCR products obtained after 2-fold step dilutions are illustrated in Figures 5.37 to 5.50. Five probes represent the TGEV genome on the array and the arrays were able to detect TGEV in all diluted samples (Green spots) albeit not with all probes. Two TGEV probes in particular (TGEV_0166 and TGEV_0168) performed well, with relatively high signal intensities (0.40 to 0.88) throughout the hybridisation assay to the array. The other probes did not perform well on this occasion in contrast to earlier studies in this chapter, for example the comparison between specific and random amplification (Fig. 5.22), where random amplified targets hybridised with higher signal intensities. The hybridisation was positive for these two probes with the minimum copy number (2.420×10^5) attempted here. The signal intensity decreased progressively with the level of serial dilution for TGEV_0168, whereas it remained relatively high throughout the decreasing copy number for TGEV_0166. The three other probes (TGEV_1, TGEV_2 and TGEV_0163) performed poorly with signal intensities lower than the detection limit, around the background intensity. Cross-hybridisations with other probes on the array were also observed, in particular with Porcine kobuvirus (P_kob_6). Other probes (Bov_cor_0229, Bov_cor_0229, Bov_tor_0044 and PEDV_2) showed positive hybridisation with TGEV target which however, can be attributed to the presence of shared genes as they are all members of the family *Coronaviridae*.

Porcine rotavirus A probe Por_roA_3 gave a positive hybridisation with all tested arrays in the sensitivity test assay with PCV-2 and TGEV.

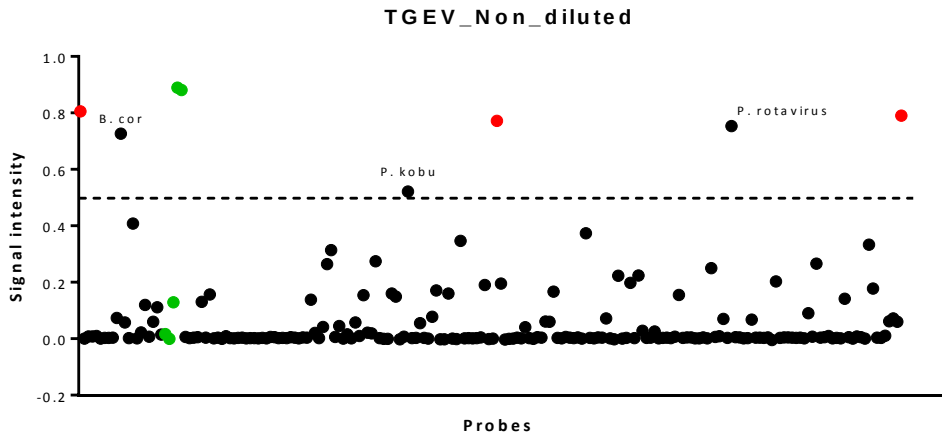


Figure 5.37. Normalised signal intensities of hybridised non-diluted TGEV (61.61 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.50 is represented by a dashed horizontal line. The corresponding copy number in the sample was 3.823×10^9.

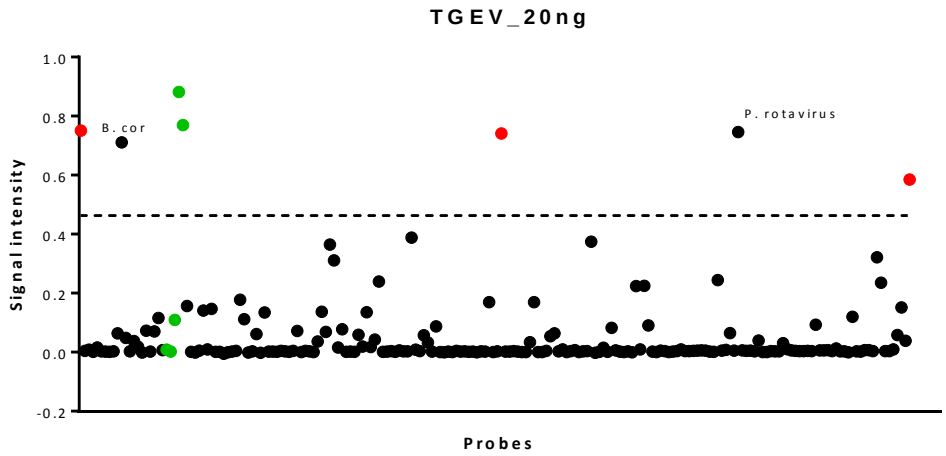


Figure 5.38. Normalised signal intensities of hybridised TGEV (20 ng/µl)

Red spots (A) and red letter B (B) correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.47 is represented by a dashed horizontal line. The corresponding copy number in the sample was 1.241×10^9.

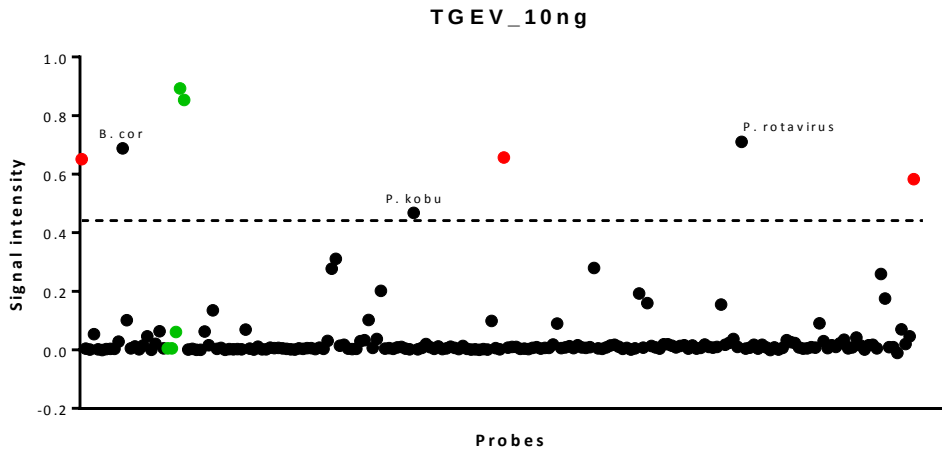


Figure 5.39. Normalised signal intensities of hybridised TGEV (10 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.44 is represented by a dashed horizontal line. The corresponding copy number in the sample was 6.205 x 10^8.

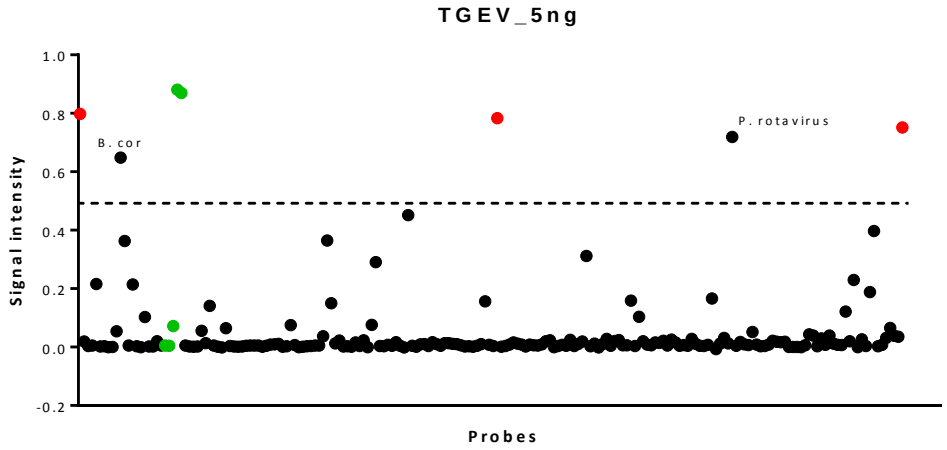


Figure 5.40. Normalised signal intensities of hybridised TGEV (5 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.49 is represented by a dashed horizontal line. The corresponding copy number in the sample was 3.103 x 10^8.

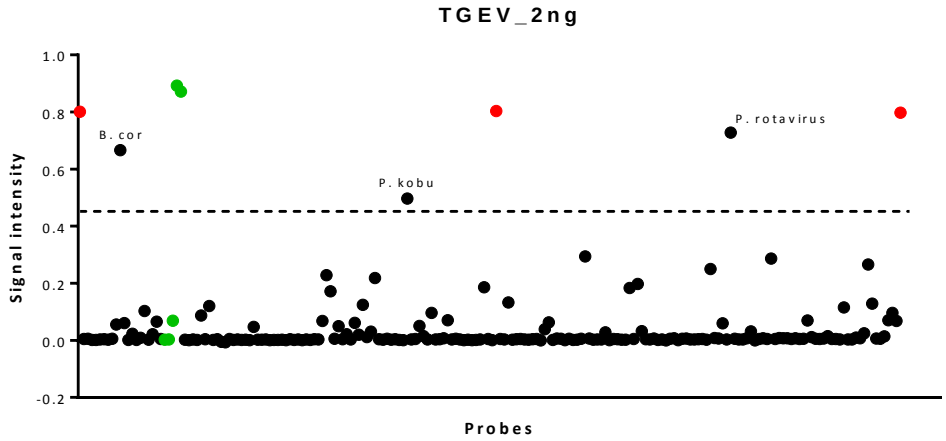


Figure 5.41. Normalised signal intensities of hybridised TGEV (2 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.45 is represented by a dashed horizontal line. The corresponding copy number in the sample was 1.241 x 10^8.

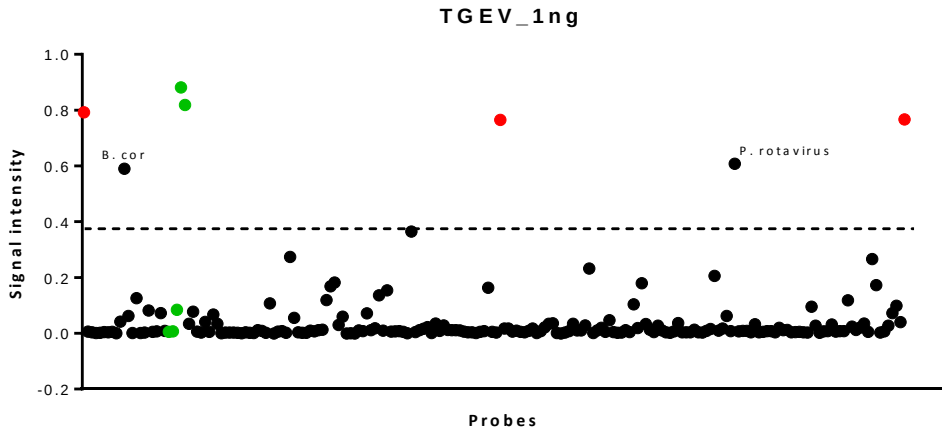


Figure 5.42. Normalised signal intensities (of hybridised TGEV (1 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.37 is represented by a dashed horizontal line. The corresponding copy number in the sample was 6.205 x 10^7.

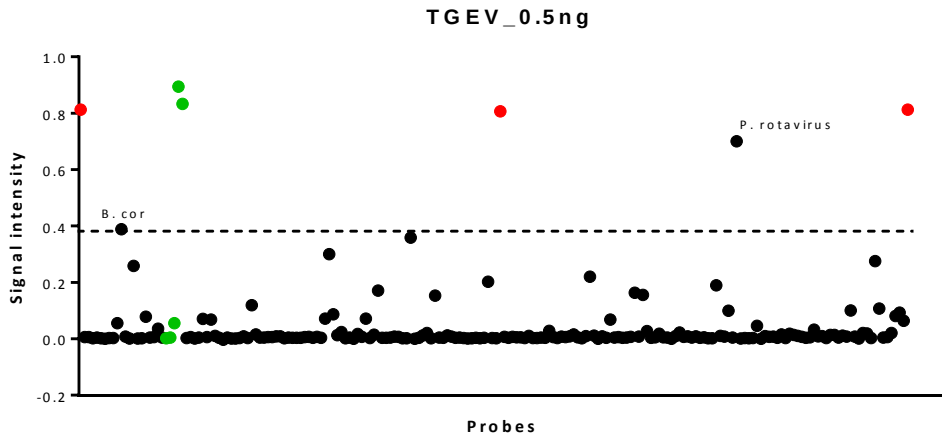


Figure 5.43. Normalised signal intensities of hybridised TGEV (0.5 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.38 is represented by a dashed horizontal line. The corresponding copy number in the sample was 3.103×10^7.

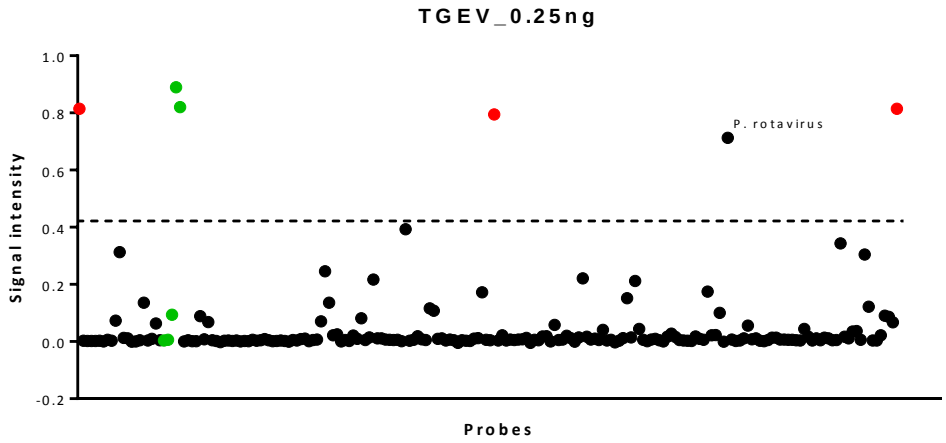


Figure 5.44. Normalised signal intensities of hybridised TGEV (0.25 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.42 is represented by a dashed horizontal line. The corresponding copy number in the sample was 1.551×10^7.

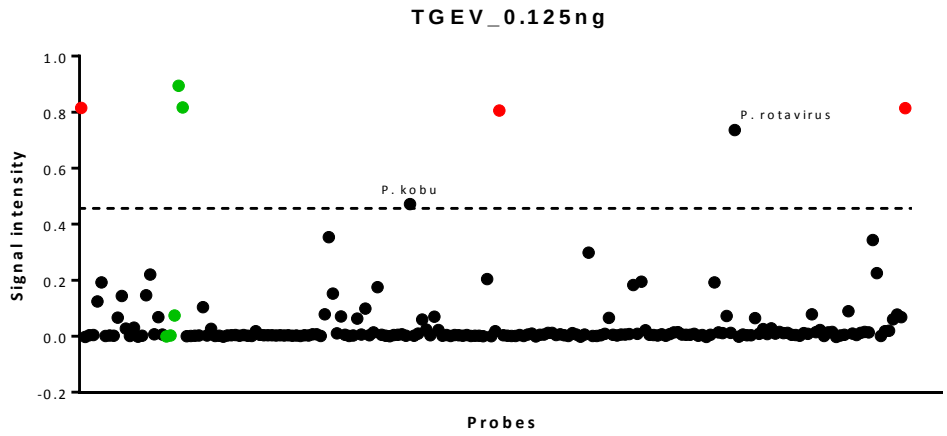


Figure 5.45. Normalised signal intensities of hybridised TGEV (0.125 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.46 is represented by a dashed horizontal line. The corresponding copy number in the sample was 7.756 x 10^6.

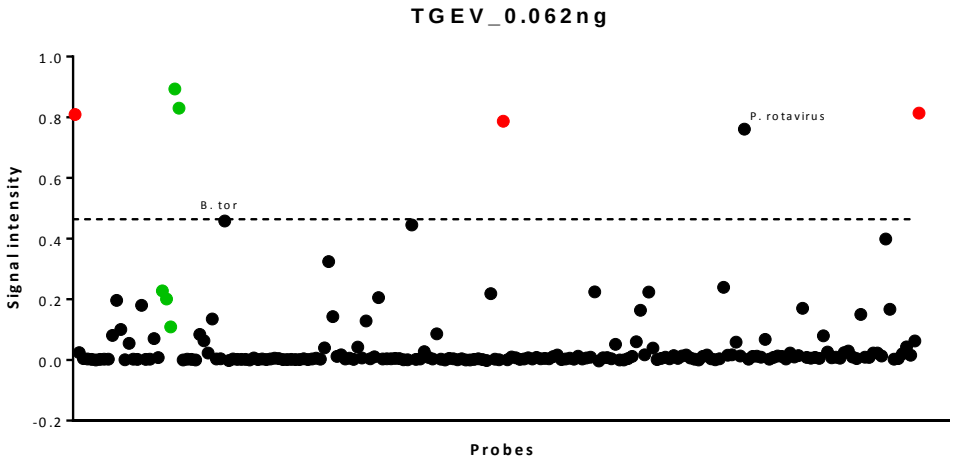


Figure 5.46. Normalised signal intensities of hybridised TGEV (0.062 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.46 is represented by a dashed horizontal line. The corresponding copy number in the sample was 3.847 x 10^6.

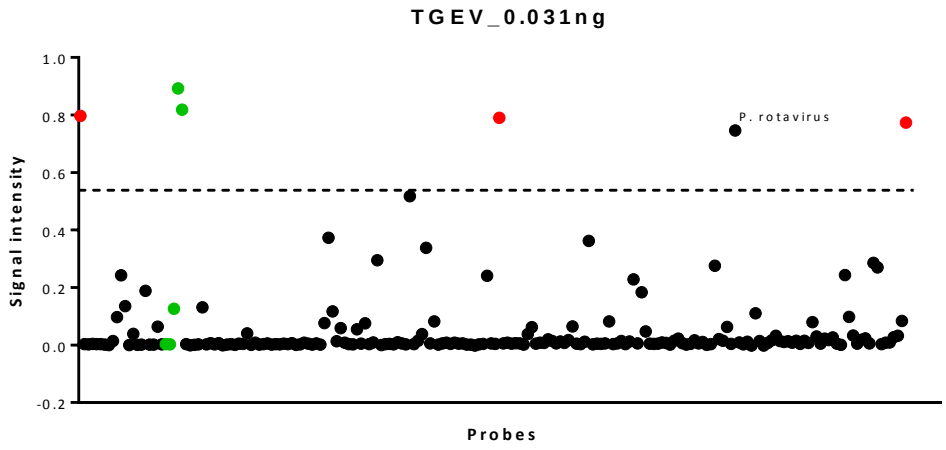


Figure 5.47. Normalised signal intensities of hybridised TGEV (0.031 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.54 is represented by a dashed horizontal line. The corresponding copy number in the sample was 1.924 x 10^6.

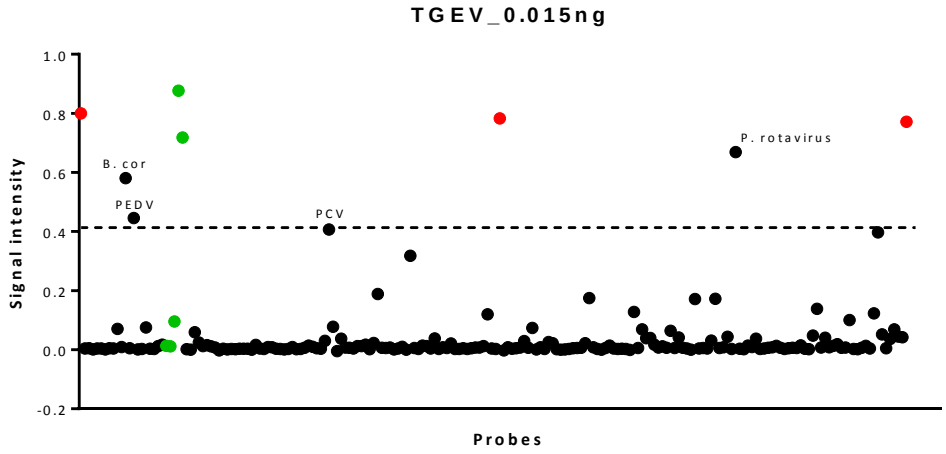


Figure 5.48.Normalised signal intensities) of hybridised TGEV (0.015 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.41 is represented by a dashed horizontal line. The corresponding copy number in the sample was 9.308 x 10^5.

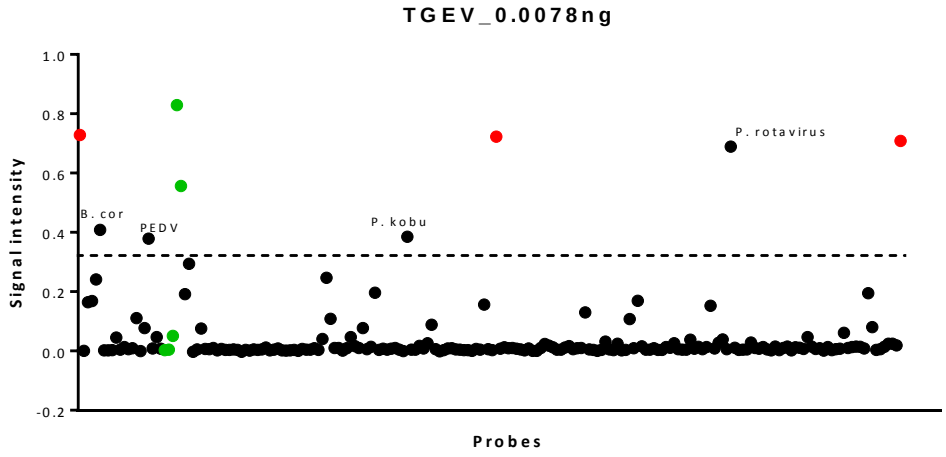


Figure 5.49. Normalised signal intensities of hybridised TGEV (0.0078 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.32 is represented by a dashed horizontal line. The corresponding copy number in the sample was 4.840 x 10^5.

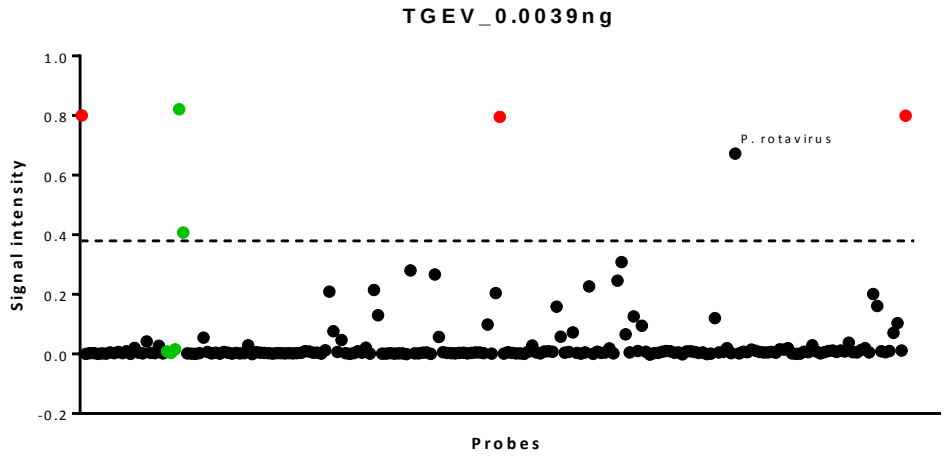


Figure 5.50. Normalised signal intensities of hybridised TGEV (0.0039 ng/µl)

Red spots correspond to Biotin markers; Green spots correspond to TGEV. Signal cut-off of 0.38 is represented by a dashed horizontal line. The corresponding copy number in the sample was 2.420 x 10^5.

Linear regression analysis between virus DNA amounts and signal intensities data of the corresponding probes was determined. Eight probes and 2 probes corresponding to the hybridised PCR product to the array respectively for PCV and TGEV. The PCV curve (Fig. 5.51), indicated a significant non-zero slope with a R^2 value of 0.62. The curve suggests consistency between signal intensity and dilution, indicating a degree of reproducibility between repeat samples.

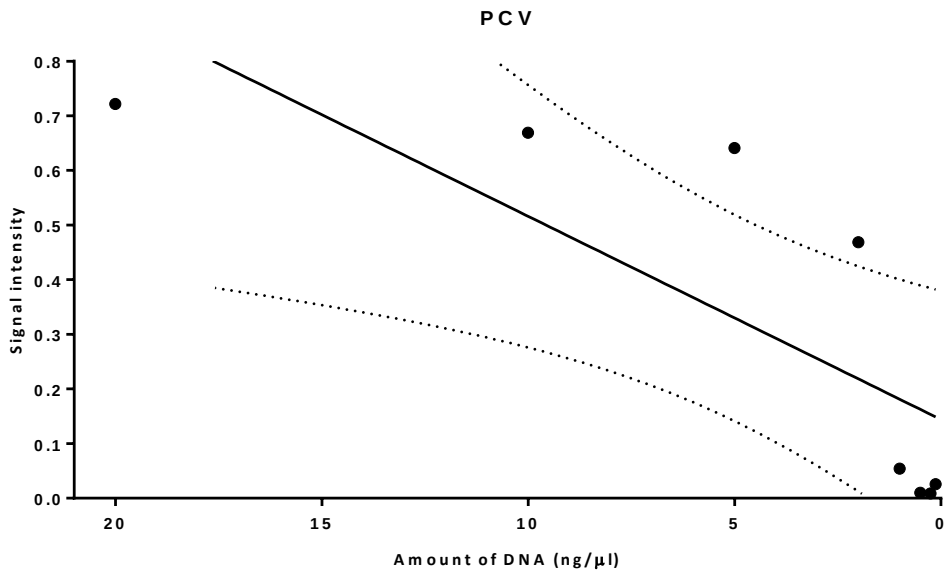


Figure 5.51. Median signal intensity of PCV PCR-specific probes of decreasing amount of DNA

Array hybridisation demonstrated an agreement between descending load of the virus and decreasing signal intensity with a coefficient of determination (R^2) of 0.62.

This was not the case with TGEV (Fig. 5.52). The curve indicated saturation for some of the dilution range and the values for the four highest dilutions examined did not show a reasonable slope indicationg either that an insufficient number of dilutions were used or that the reproducibility was poor.

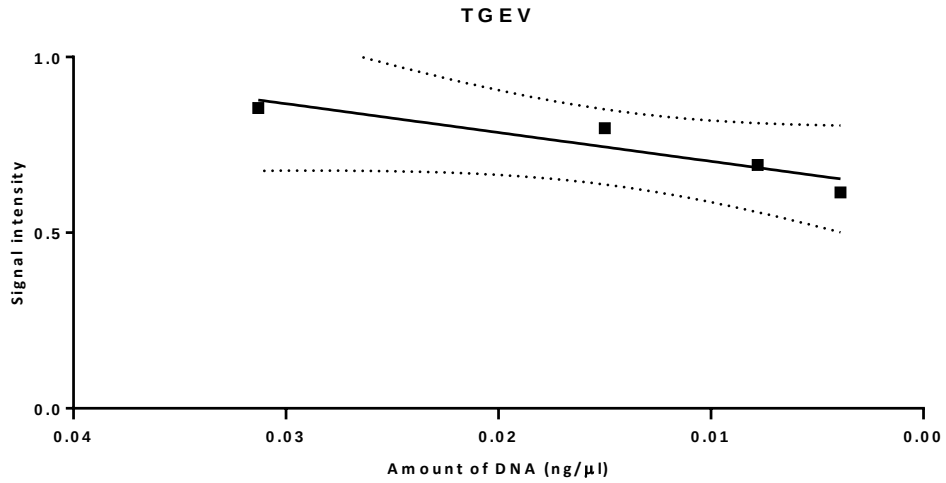


Figure 5.52. Median signal intensity of TGEV PCR-specific probes of the four highest DNA dilutions.

The signal intensity decreased with descending amount of TGEV DNA with a relatively high coefficient of determination (R^2) of 0.86.

5. 3. 3. Array hybridisation with clinical samples

Independent array hybridisations were performed with four pig faecal samples and 12 calf faecal samples.

5. 3. 3. 1. Porcine clinical samples

The same porcine samples were subjected independently to random amplification and specific amplifications, the latter to act as a reference with which to compare the results of random amplification followed by biotin labelling (Appendix XIV).

a. Random amplified samples

Figures 5.51 and 5.52 show the signal intensities of the four samples. The results of random amplification of the samples clearly showed different patterns in the four samples. Sample 7 appeared to show positive hybridisation of PEDV and TGEV probes, sample 13 also showed positive signal intensities mainly for TGEV probes whereas sample 14 had positive signals for two probes for PEDV and four for TGEV. Sample 8 had the highest number of different positive probes, it showed the presence of PCV, Bovine torovirus, sapovirus, enterovirus and norovirus. Samples 13 and 14 were positive to PCV and Porcine norovirus. Sapovirus and Porcine rotavirus A were positive only in sample 14. As noted previously, the probe Por_rotA_3 (corresponding to P. rotavirus A - labelled in the figures) was positive in the four samples.

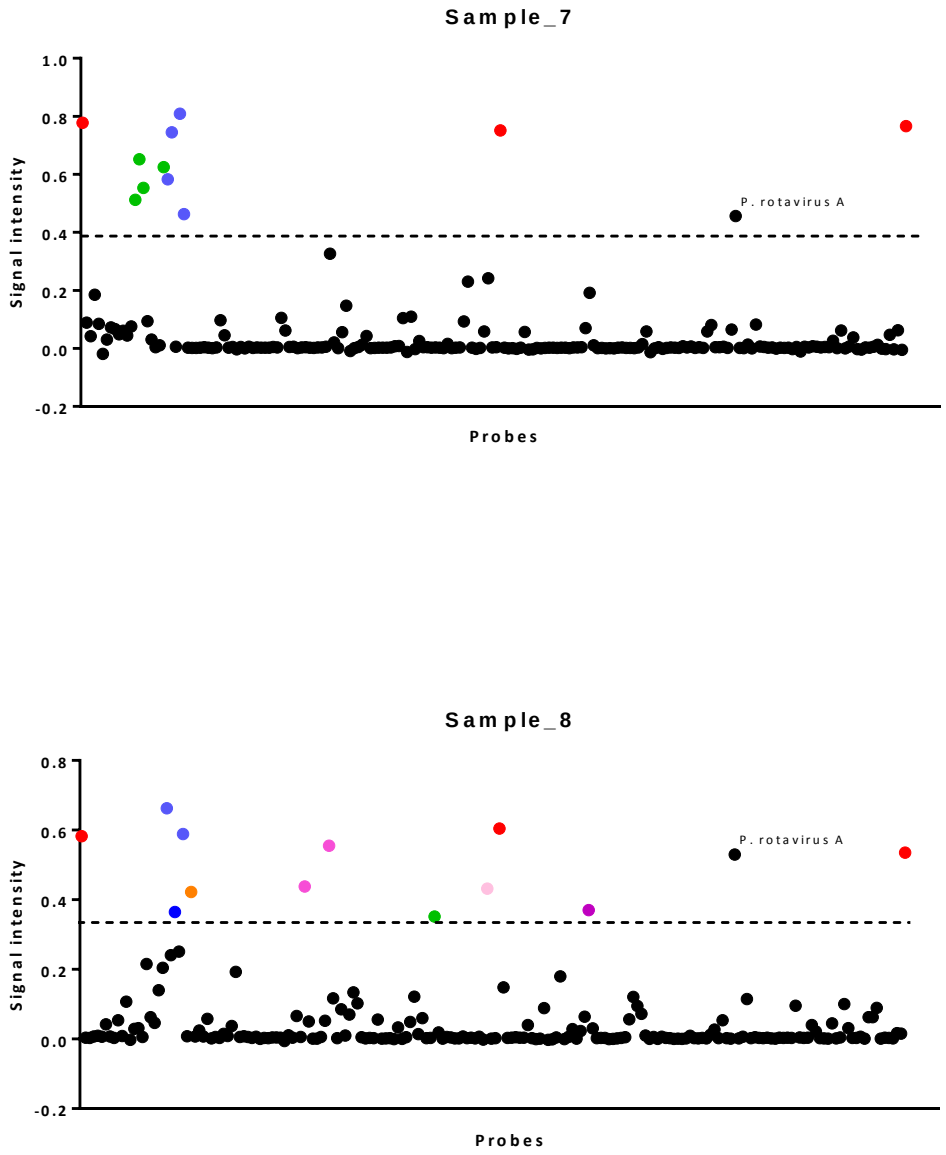


Figure 5.53. Normalised signal intensities of hybridised porcine clinical samples 7 and 8 following random amplification

Red: Biotin markers, Blue: TGEV, Green: PEDV, Orange: B. torovirus, Pink: PCV, Grey, B. enterovirus, Light pink: Sapovirus, Purple: P. norovirus. Signal cut-off of 0.39 and 0.34 for samples 7 and 8 respectively are represented by a dashed horizontal line. TGEV probes showed high signals for sample 7 and 8 (4 probes and 3 probes out of 5 respectively), PEDV probes have also hybridised to the target in sample 8, however this result was not confirmed by PCR.

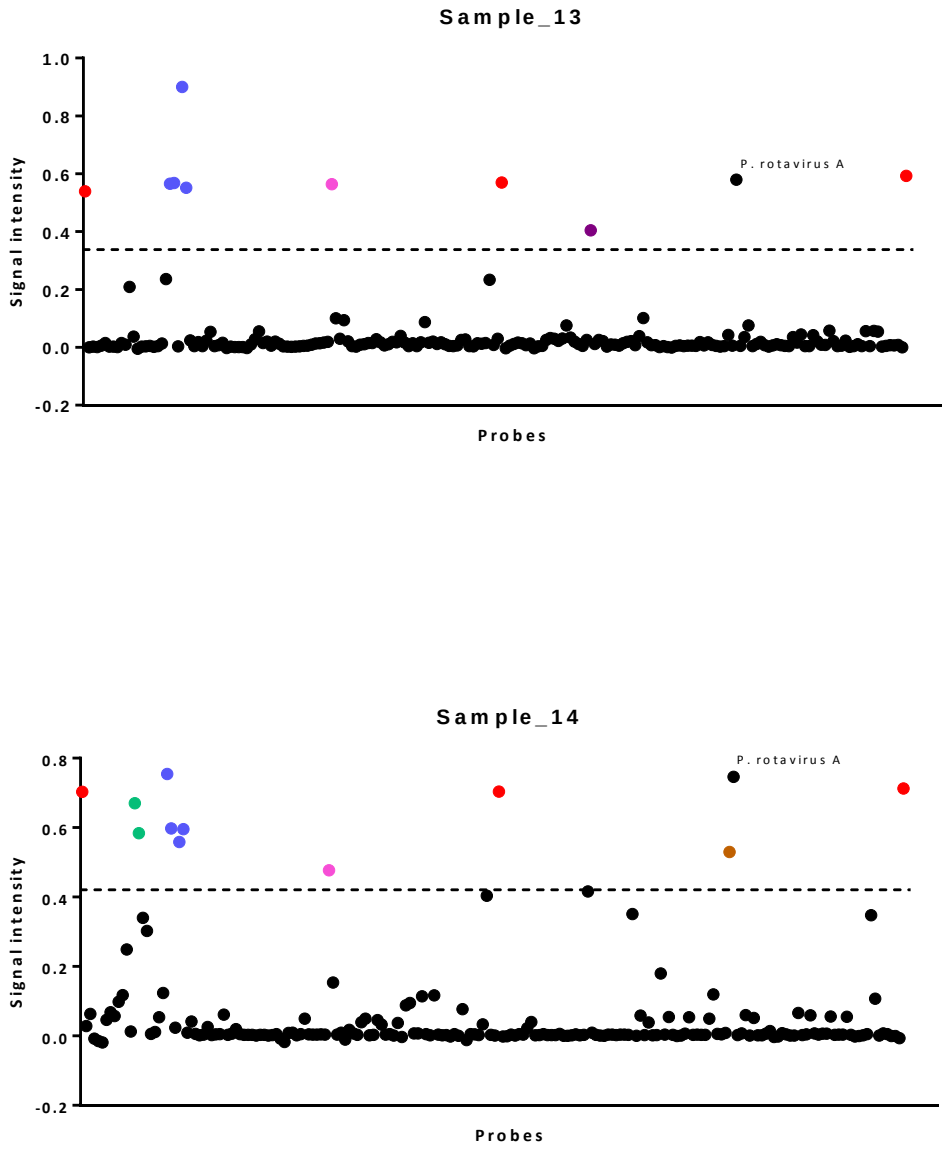


Figure 5.54. Normalised signal intensities of hybridised porcine clinical samples 13 and 14 using random amplification

Red spot: Biotin markers, Blue spot: TGEV, Green spot: PEDV, Pink spot: PCV, Purple spot: P. norovirus, Brown spot: P. rotavirus A. Signal cut-off of 0.34 and 0.42 for samples 13 and 14 respectively are represented by a dashed horizontal line. Samples 13 and 14 showed the presence of TGEV (4 out of 5 in both samples). Two PEDV probes had also high signal intensities. The PCR did not show the presence of these two viruses.

b. Sequence-specific amplified samples

The same porcine clinical samples previously used in the random amplification were used to amplify specific fragments of Porcine kobuvirus and Porcine rotavirus. P. rotavirus and P. kobuvirus were detected by multiplex PCR using a set of designed and published primers, as shown in figure 5.53. This indicated that samples 8, 13 and possibly also 7 and 14 contained P. kobuvirus and samples 7, 8 and 14 contained P. rotavirus.

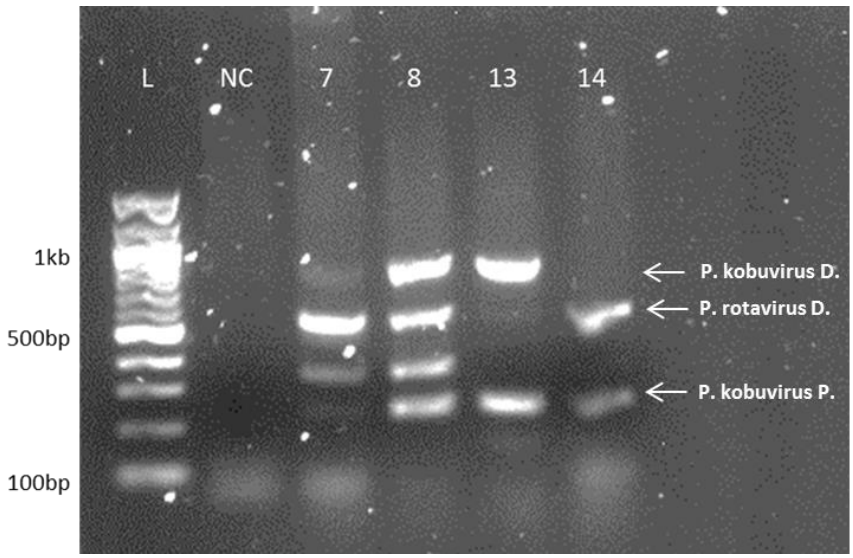


Figure 5.55. Agarose gel electrophoresis of multiplex PCR detection of Porcine kobuvirus and Porcine rotavirus

L: 100 bp ladder, NC: Negative control, 7-14: porcine clinical samples.
PCR product size:
Porcine kobuvirus designed (D) primers (VP1), labelled P. kobuvirus D.: 803 bp
Porcine kobuvirus published (P) primers (RdRp), labelled P. kobuvirus P.: 216 bp
Porcine rotavirus A designed (D) primers (VP7), labelled P. rotavirus D.: 521 bp
Porcine rotavirus A published (P) primers (VP7): 1062 bp

The signal intensity after hybridisation of the above samples are illustrated in Appendix XV. High signal (Fig. 5.54, and 5.55) showed the presence of P. kobuvirus in samples 7, 8 and 13 (amplified with designed primers) (blue dots for P. kobuvirus and light blue for B. kobuvirus). This finding was already confirmed by multiplex PCR although this was less clear for sample 7 (Fig. 5.53). Even though a strong band was seen in the PCR gel in sample 14 for P. rotavirus, this was not confirmed by the array. None of the samples showed strong signals for rotavirus, except from sample 8, where an average signal of 0.50 for two specific probes to the porcine virus were registered. The other probes giving signals just above or below the cut-off value. Additional spots from other viruses including P. torovirus, P. enterovirus, BVDV, P. bocavirus and P. parvovirus showed intensities slightly above the cut-off value.

In sample 7, the biotin markers showed a low signal intensity which should vary between 0.70 to 0.80. It is only in this array that the signals were 0.19-0.25, however the array successfully detected P. kobuvirus, as it was confirmed by PCR.

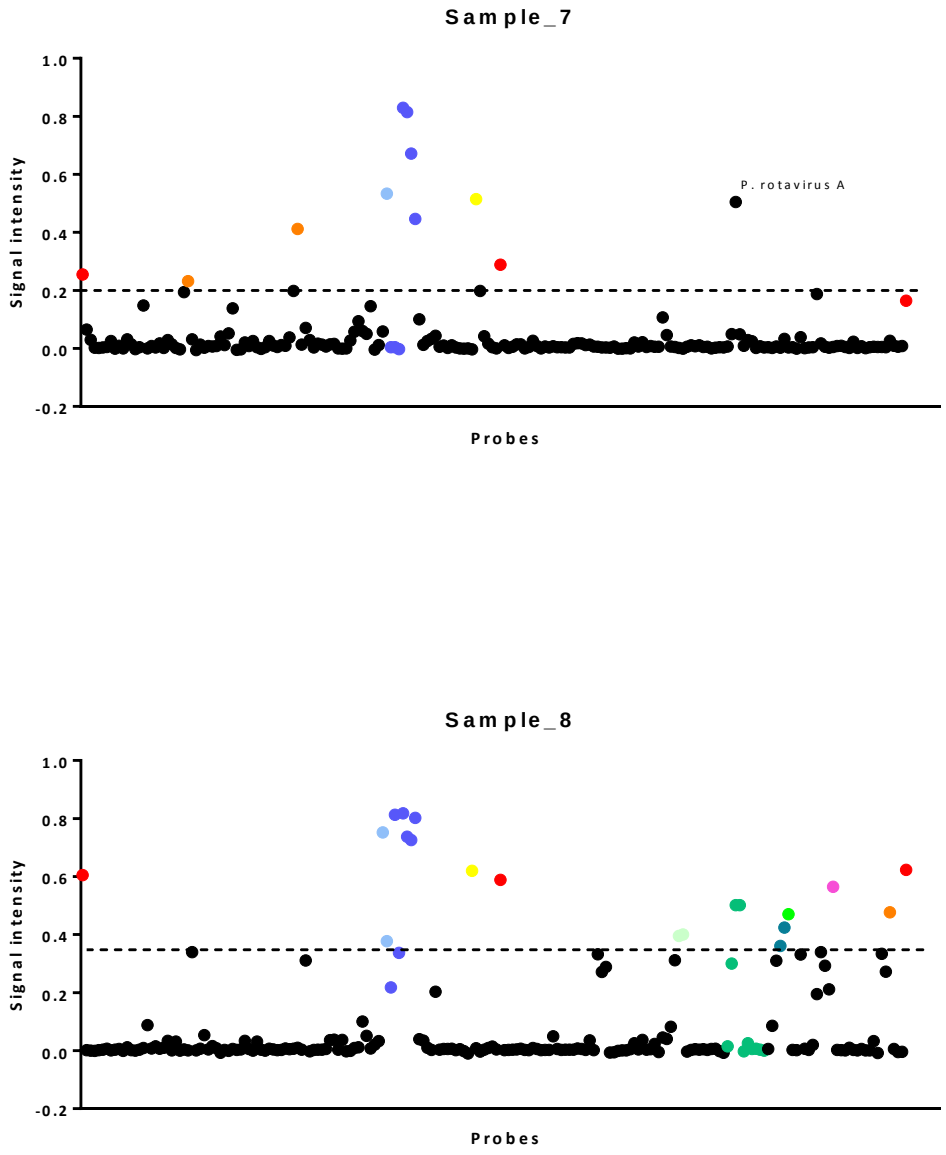


Figure 5.56. Normalised signal intensities of hybridised porcine clinical samples 7 and 8 using multiplex amplification (samples 7 and 8 in Fig. 5.53)

Red spot: Biotin markers, Blue spot: P. kobuvirus, Light blue spot: B. kobuvirus, Yellow spot: P. enterovirus, Green spot: P. rotavirus A, Light green spot: B. rotavirus A, Dark green spot: P. rotavirus B, Lime green spot: P. rotavirus C, Pink spot: BVDV2, Orange spot: P. bocavirus. Signal cut-off of 0.2 and 0.35 respectively for sample 7 and 8 are represented by a dashed horizontal line. Sample 7 and 8 show a positive hybridisation to P. kobuvirus probes, although the expected PCR product for P. kobuvirus was weakly apparent (Fig.5.53). Surprisingly, none of P. rotavirus probes hybridised, despite a strong band in the gel showing the presene of the virus in the sample. Sample 8 hybridisation results and multiplex PCR results are in accordance, as both show the presence of P. rotavirus and P. kobuvirus.

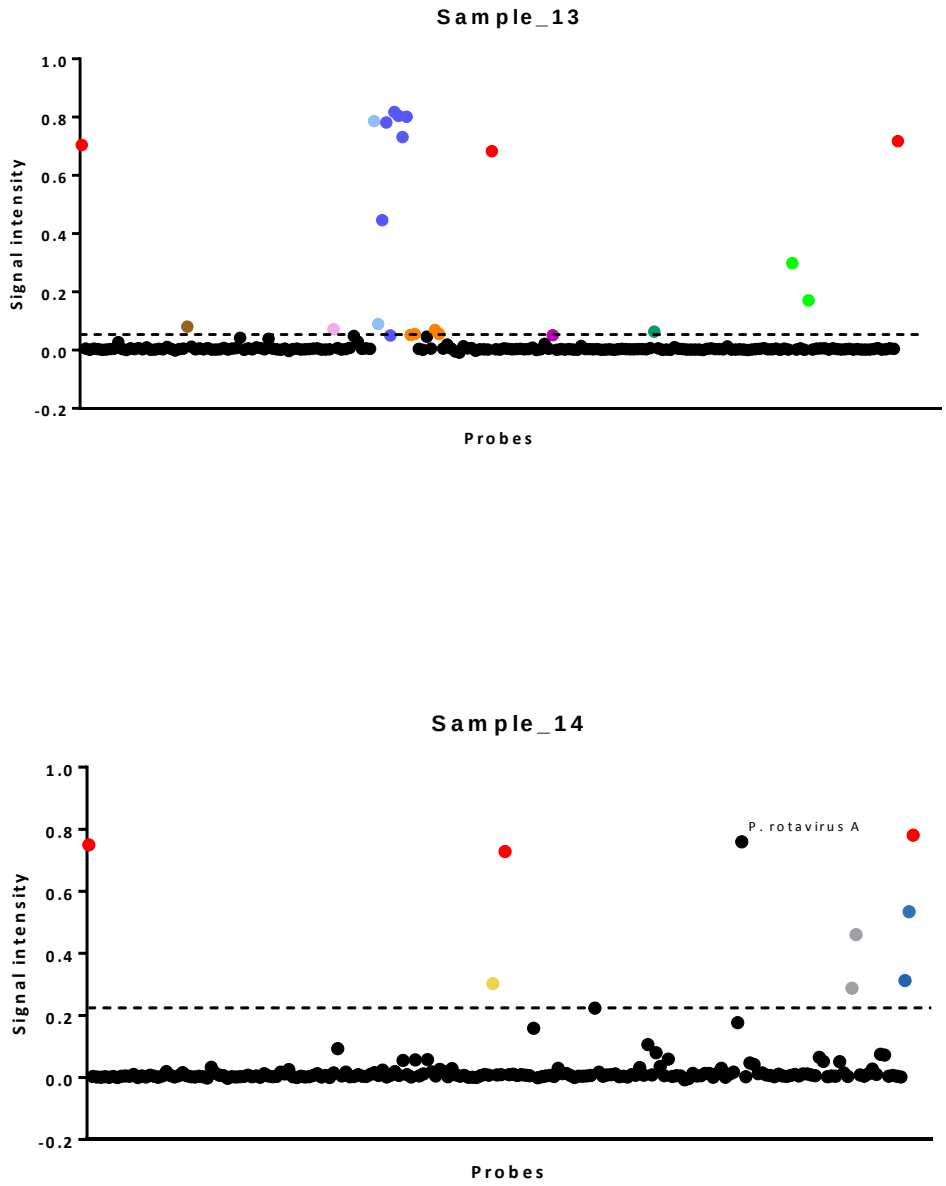


Figure 5.57. Normalised signal intensities of hybridised porcine clinical samples 13 and 14 using multiplex amplification (samples 13 and 14 in Fig. 5.53)

Red spot: Biotin markers, Blue spot: P. kobuvirus, Light blue spot: B. kobuvirus, Brown spot: B. torovirus, Pink spot: PCV, Orange spot: P. enterovirus, Purple spot: B. norovirus, Green spot: B. rotavirus A, Limegreen spot: P. rotavirus C, Yellow spot: P. sapelovirus, Grey spot: BVDV, Navy blue: P. parvovirus. Signal cut-off of 0.051 and 0.23 respectively for sample 13 and 14 are represented by a dashed horizontal line.

Sample 13 contains P. kobuvirus only, according to the multiplex PCR, it showed that seven P. kobuvirus-specific probes hybridised with high signal among a total of 7 probes on the array. Other probes signals were slightly above the cut-off limit. Sample 14 shows no specific probes to P. kobuvirus or P. rotavirus despite the presence of P. rotavirus in the sample. P. kobuvirus was evidenced by PCR using published primers only.

5. 3. 3. 2. Bovine clinical samples

Twelve bovine clinical samples consisting of positive samples to one or more targeted pathogens identified by multiplex PCR, were labelled after a random amplification, then hybridised to the AT platform. The results of hybridisation signal intensities above the cut-off value of the 12 samples are illustrated in Table 5.6 (cut-off indicated in the bottom column). The animals from which the samples were taken were diarrhoeic and a number of probes generated signals above the cut-off but none of them was particularly high with perhaps the exception of samples 13, 19.1, 25T, 55 and B2 where the signals varied between 0.50 and 0.82. In these same samples, signals above the detection limit showed the presence of PEDV (obviously cross-hybridising with Coronavirus genome) for sample 13, norovirus for samples 19.1, 25T and 55, kobuvirus for sample B2 and Coronavirus for sample 25T. In most samples, targets hybridised to few probes with intensities slightly over the cut-off. Ten samples out of 12 showed positive hybridisation with one to two norovirus probes, which indicates that the virus was present as it was detected in 10 samples out of 12. Also, the signal intensities seem to indicate that in all the samples, two to three genetically distant species of viruses were present, considering that probes can hybridise to similar genome regions of viruses, belonging to genera of the same family, or again to the same genus, but host-specific viruses.

Table 5.4. Signal intensities above the cut-off of bovine clinical samples after specific amplification and hybridisation to the ArrayTube

Probes	1/3	6T	9T	13	19.1	1	5/8	19.3	20T	25T	55	B2
Bov Cor 0231	-	-	-	-	-	-	-	-	0.330706	0.82129	-	-
PEDV_2	-	-	-	0.652748	-	-	-	-	-	-	-	-
TGEV_1	-	-	-	-	-	0.069363	-	-	-	-	-	-
Bov tor_2	0.457790	-	-	-	-	-	-	-	-	-	-	-
Por tor_4	0.273196	-	-	-	-	-	-	-	-	-	-	-
Por tor_8	-	-	-	-	-	0.059333	-	-	-	-	-	-
Por cir 0529	-	0.410279	-	-	-	-	-	-	-	-	-	-
Bov kob_1	-	-	-	-	0.135849	-	-	-	-	-	-	-
Bov kob 0008	-	-	0.07249	-	-	-	-	-	-	-	-	-
Por kob_2	0.169803	-	-	-	-	-	-	-	-	-	-	-
Por kob_7	-	-	-	-	-	-	-	-	-	-	-	0.601767
Bov ent_5	-	-	-	-	-	-	-	-	-	-	-	0.352354
Por sap_1	-	-	-	-	0.148037	-	-	-	-	-	-	-
Por tes_2	-	-	-	-	-	-	-	0.07299	-	-	-	-
Neb_1	-	-	-	-	-	0.090175	-	-	-	-	-	-
Neb_3	-	-	-	-	-	0.079466	-	-	-	-	-	-
Sap_1	-	-	-	-	-	-	0.328633	-	-	-	-	-
Sap_5	-	-	-	-	-	-	-	-	-	-	0.141356	-
SI Cut-off	**0.15**	**0.17**	**0.05**	**0.15**	**0.16**	**0.04**	**0.09**	**0.07**	**0.08**	**0.21**	**0.14**	**0.3**

SI: Signal intensity

Continued

Probes	1/3	6T	9T	13	19.1	1	5/8	19.3	20T	25T	55	B2
Bov nor_3	-	-	-	-	-	-	-	-	-	-	0.515724	-
Bov nor_6	-	-	-	0.13904	-	-	-	-	-	-	-	-
Bov nor_7	-	0.206271	-	-	-	-	0.126667	-	-	0.505862	-	-
Bov nor_9	-	-	-	-	-	-	-	0.211044	-	-	-	-
Bov nor_10	-	-	-	-	-	-	-	0.152611	-	-	-	-
Por nor_6	0.305153	0.215609	0.109794	-	0.701679	-	0.129031	-	-	-	-	0.382684
Bov rot A_1	-	-	-	-	-	-	0.150204	-	-	-	-	-
Bov rot A_9	-	-	0.067178	-	-	-	-	-	-	-	-	-
Bov rot A_10	-	-	0.143591	-	-	-	-	-	-	-	-	-
Por rot A_4	-	-	-	-	-	0.051949	-	-	-	-	-	-
Por rot C_5	-	-	-	-	-	-	-	-	-	-	-	0.350937
Bov par_2	-	-	-	-	-	-	-	-	-	-	0.28329	-
SI Cut-off	**0.15**	**0.17**	**0.05**	**0.15**	**0.16**	**0.04**	**0.09**	**0.07**	**0.08**	**0.21**	**0.14**	**0.3**

SI: Signal intensity

5. 4. Discussion

The studies in this chapter indicate the importance of good probe design followed by their validation with reference samples. The experiments on specificity and cross-hybridisation highlighted that a number of the probes used had weaknesses and in the normal course of events and in future studies would be removed or replaced where they were found to be unsufficiently sensitive or specific or where their performance was inconsistent, although it is suspected that this latter characteristic probably more accurately reflects a need for greater stringency in the conditions of the PCR process. The TGEV probes are a case in point where under some circumstances they performed well, for example when tested against single reference samples, but did less well when tested in a mixture of pathogens. They also performed well when samples were diluted but in this situation not all the probes performed similarly well.

A number of aspects of array strengths and weaknesses were assessed in a preliminary way to begin to determine how far arrays of this sort might be used for diagnosis and surveillance.

Specificity of the ArrayTube

The need for rational probe design and elimination of ineffective probes was indicated initially by the fact that ArrayTube hybridisation, using the Alere platform with reference viral species following random amplification, showed that the probes on the array were able to identify the targets of five among eight tested viruses. PEDV, TGEV, PCV-2, BVDV and PPV were identified

correctly. However, Porcine rotavirus A, Porcine bocavirus and Porcine torovirus were not detected. Only two probes out of 9 were positive for BVDV. It is important to note that only PEDV, TGEV, PCV-2 and P. rotavirus A had their identity confirmed by monoplex PCR with primers designed in this study, while monoplex PCR using published primers was performed on Porcine bocavirus and parvovirus only. In terms of discrepancies between the PCR and the microarray, the results indicate that P. rotavirus A, P. bocavirus and B. kobuvirus were not detected with the array, all of which were detected by PCR, either using published or designed primers. This indicates that PCR technique is a valuable tool for the detection of a sequence in a sample, with primer specificity being an important component of the performance of the PCR reaction, but it also suggests that these samples might have contained a viral load below the detectable range of the Alere array.

Another important aspect is the quality of the sample is an important element in the detection process, meaning that at this stage of the array development, and particularly at the probes level, it is inappropriate to attribute the lack of detection of the viral DNA/cDNA to the probe design or the microarray platform, in this case, specifically for P. torovirus, a donated virus, which identity was not verified).

Similarly the use of mixed nucleic acid species using two sets of multiplexed reference strains, with set 1 including TGEV, PCV2, *S.* Typhimurium, *S.* Enteritidis and *C. difficile* (Multiplex_1) and set 2 including *B. pilosicoli*, PEDV, Porcine rotavirus A, *E. acervulina* and *C. jejuni* (Multiplex_2), showed that the

array was able to identify the two viruses in Multiplex_1 mixture, whereas it was only capable of detecting PEDV viral genes in Multiplex_2, and then with only half of the probes. Probes of Porcine rotavirus demonstrated very weak signal intensities that remained below the cut-off signal. A high level of noise was observed in this array, probably due to the presence of similar regions with DNA of amplified products from the 16S/18S sequence of *B. pilosicoli*, *E. acervulina* and *C. jejuni* with probes. It has been shown elsewhere that increasing the complexity of the sample could lead to higher levels of cross-hybridisation (Wick et al., 2006).

The assessment of non-targeted viral species did show a degree of cross contamination, more with Bunyamwera and Schmallenberg virus than equine influenza, again emphasising the need for good probe design and elimination of probes which generate cross hybridisation. Cross-hybridisation generally occurs when probes share high sequence similarity with a non-target molecule; it thus depends on the sequence composition of the DNA of the hybridised pathogen. BLAST search of sequence similarity for sequences from the three viruses did not show an alignment with any of the viruses represented on the array. However, considering the length of the sequences tested, it is unquestionably preferable to check the specificity of each probe against all sequenced genomes in the database. Eventually probes showing high similarities with related and non-related organisms, or unexpectedly exhibiting non-specific hybridisation should be removed from future array work/design

It is important to note that the high background noise generated by the hybridisation of Bunyamwera and Schmallenberg virus impacted the cut-off which exceeded the biotin markers values, which rises the question whether these assays are valid.

A certain level of cross hybridisation occurred with cytochrome b than with a negative sample, although below the signal cut-off limit. It may be that a single gene present in high amounts could give an unbalanced view of likely host genes present, whereas the negative gut sample represents a wide range of host genes generally present in much lower quantities. The cross hybridisation observed with cytochrome b may therefore be assessed as less relevant than a truly negative gut sample.

One probe P_rotA_3, was positive in nearly all arrays carried out. Similarly a relatively high signal intensity (0.34) was observed in the negative control (nuclease-free water as template), which might indicate possible biotin cross-contamination during the spotting process of the microarray.

Specificity and Cross hybridisation

On the array, a 60-mer probe sharing more than 70 to 75% identity with non-target sequences might lead to significant cross-hybridisation (Kane et al., 2000; Hughes et al., 2001). Again because, long probes tolerate more mismatches than short probes, partial hybridisation to non-target molecules can generally lead to false positives. At the molecular level, a spot is a combination of a multitude of identical probes that can hybridise to multiple targets with perfect matches and mismatches, or one single probe can perfectly

hybridise to a single target or with mismatches (Koltai and Weingarten-Baror, 2008). In both cases, hybridisation of a fraction of the probe can generate a signal that may dramatically impact the specificity of the microarray.

The specificity of the microarray signifies whether it can identify correctly targeted pathogens, it equally signifies that it should not identify a pathogen that is not represented by the probe sets, unless it is related to that pathogen. Cross hybridisation could also arise from a mere conformational interaction between DNA molecules irrespective of sequence relatedness. This is to ensure that the array delivers accurate and non-ambiguous results. Many microarray detection studies have reported false positive results where cross-hybridisation with non-intended species occurred (Volokhov et al., 2002; Leinberger et al., 2005; Wong et al., 2007; Järvinen et al., 2009)

Specific versus random amplification

Random amplification is an alternative to specific amplification for amplifying targets when small amounts of nucleic acids are extracted from faecal material. This method allows the amplification of highly divergent sequences without prior knowledge of the microorganism sequence. However, as the amplification requires the use of random primers, their binding to any nucleic acid present in the sample is not governed by specificity. Consequently, the most highly representative amplicons may be from the host, commensal microrganisms rather than from enteric pathogens depending on the nature of the sample e.g. faeces. One of the disadvantages of this amplification method is that accidental cross contamination could lead to false positives resulting

from contamination while performing any nucleic acid extraction, reverse transcription or amplification procedure, although this did not appear to be a major issue. Furthermore, contamination can conceivably arise following the addition of each reagent (Kotilainen et al., 1998).

Two viruses, the TGEV and PCV-2 reference strains, were used to compare the impact of random and specific amplification on the outcome of the array hybridisation. Contrary to what was expected, our results showed that random amplification was the best amplification strategy for PCV and TGEV hybridisation. Higher number of virus-specific probes hybridised with signals above the cut-off for both viruses. In the specific amplification approach, fewer probes corresponding to the hybridised viruses registered signals above the cut-off, however the intensity of the signal was remarkably higher than the intensity of the probes when random hybridisation was carried out. This could be explained by the characteristic of the PCR reaction that exponentially amplifies a sequence of gene resulting in a considerable number of copies of the same sequence which only favours probes that hybridise to the amplified gene, whereas in the random amplification more probes have nearly the same chance to hybridise to their targets. For both viruses, only probes that hybridised to amplified genes were positive in the specific amplification with 40% and 42% of TGEV and PCV-2 probes that gave positive hybridisation respectively, in the specific amplification, as opposed to 100% and 75% of positive probes in random amplification.

In the amplification strategy adopted in this study, the PCR products obtained were subjected to random amplification to perform the biotin-labelling and this step might have contributed to increasing the amount of non-specific targets in the sample. This could have been further investigated by the use of biotin-labelled primers to obviate the need for the random amplification step, but would have been costly. In one study, using the same microarray platform as used in this study, amplified pathogen targets with biotinylated primers, resulted in comparable sensitivity to PCR, but less sensitivity was observed compared with real time PCR (Giles et al., 2015).

Several other studies indicated that the use of amplification with random primers as an amplification strategy resulted in low sensitivity rates compared to specific PCR (Vora et al., 2004). Also in the study of Lin et al. (2006) the use of specific amplification for clinical samples increased the sensitivity of the microarray whereas random amplification showed a low sensitivity and high specificity. However, in the latter study samples were treated for host DNA removal prior to amplification.

It has already been demonstrated that microarray hybridisation using specifically amplified products allowed highly sensitive and specific pathogen identification (Wilson et al., 2002). In this study the background noise in the random amplifications was much lower than in the specific.

Sensitivity

The sensitivity of the AT platform was assessed using two viruses PCV-2 and TGEV. Two-fold dilutions of total nucleic acid of the reference strains were

amplified by PCR, then biotin-labelled and hybridised to the probes. Array results were then compared with the PCR reactions observed by gel electrophoresis. Gel electrophoresis analysis of PCV PCR products, showed that it could detect down to 2.065 x 10^9 copy number, meaning that the gel is able to detect the presence of the virus in a sample containing at least 2 ng of PCV genomic DNA. For TGEV, gel electrophoresis bands could be detectable clearly at a concentration of 0.078 ng which corresponds in copy number to 4.84 x 10^5. Hybridisation sensitivity testing of PCV-2 showed that the sensitivity of the ArrayTube was determined to be equal to gel electrophoresis sensitivity (2ng - 2.065 x 10^9 copy number).

For TGEV, the array had a sensitivity of 0.0039 ng (39 pg) or a minimal copy number of 2.420 x 10^5 in this preparation as an initial amount of the virus in the sample. The array is expected to detect lower amounts as evidenced by the high signal intensity at the minimal copy number attempted in this study.

Linear regression was computed to determine the relationship between the copy number of both viruses and the signal intensity of the probes. A high coefficient of determination was found for PCV for most of the probes but not for TGEV, which could be explained by the difference in the number of specific probes for the two viruses. On the AT, only five probes correspond to TGEV, whereas 12 probes correspond to PCV.

The obvious reason that can explain the difference between the array sensitivity of PCV and TGEV is the primer amplification efficiency. In terms of primer design, the forward primer of PCV2 (Por cir2_F_1, Appendix IV-a) shows

that the length of the primers was 16 nucleotides, Álvarez-Fernández (2013) explained that primers shorter than 18 nucleotides perform badly with lesser specificity than 18-25 nucleotide primers. The GC content is also an important parameter in the design of primers. Imbalance in the GC content of the primers was shown to have a big influence during the annealing (Mallona et al., 2011). In this study the GC content of forward and reverse PCV2 primers were respectively 68.75% and 52.38%, compared to TGEV primers which was 55% for both primers. This also has an important effect on the melting and annealing temperature of the primers. All these conditions could explain the lack of amplification of PCV which and this might be worsened when the amount of target DNA in the sample was low.

Using a similar platform than used in this study to detect pathogens, authors reported very different detection limits, namely 1 µg for *E. coli* pathotype detection (Anjum et al., 2007), 975 ng (2.66×10^8 copies) for *Legionella pneumophila* (Żak et al., 2011), 100 genomes equivalent for four genes and 10 genomes equivalent for one specific gene of *Coxiella burnetii* (Schmoock et al., 2014).

However, the detection limit of the array might vary with the length of the probes and the amplification strategy used in the study. More sensitivity and detection limit investigations should be performed with clinical samples to objectively evaluate the performance of this array.

Clinical samples

Random amplification in porcine clinical samples (7, 8, 13 and 14) was unable to fully identify/detect the pathogens present in the samples, which were screened by multiplex PCR and confirmed to contain Porcine rotavirus A, and Porcine kobuvirus. In these samples multiplex PCR using designed and published primers led to the identification of one to two viruses in the same sample. Array hybridisation of multiplexed (specific amplification) samples 8 and 13 were in accordance with the multiplex PCR results. Sample 7 was positive to Porcine rotavirus and weakly positive to Porcine kobuvirus by PCR, whereas it was negative to Porcine rotavirus and positive to kobuvirus when tested by microarray, creating an ambiguity in the results where more sensitivity is expected from the array compared to PCR. Debaugnies et al. (2014) suggested that co-infection with a great difference in the quantity of pathogens could interfere with the analytical sensitivity of the microarray leading to a false negative for the pathogen present on lower numbers. This phenomenon has also been attributed to secondary structures of target DNA that prevent hybridisation (Peplies et al., 2003). Others have reported that secondary structures, such as hairpins, might also increase non-expected hybridisation between targets and probes (McLoughlin, 2011).

In sample 14, multiplex PCR showed the presence of Porcine rotavirus with designed primers and Porcine kobuvirus with published primers. When this sample was hybridised to the array the results were negative for both viruses. It is important to note that the amplicons of kobuvirus obtained with published

primers did not hybridise to the array, possibly due to the absence of the complementary sequences to kobuvirus probes in the amplified product, which signifies that the published primers-PCR products did not contribute to the specific array hybridisation. The presence of an unexpected band of approximatively 300 bp in the multiplex reaction of sample 8 and less visible in sample 7 possibly led to non-specific hybridisation of some probes on the array.

The twelve bovine clinical samples tested on the AT showed positive hybridisation for only one to five probes per test. Members of the family of *Caliciviridae*, in particular noroviruses may have been present in ten samples among the total number of samples. However, as these samples were amplified by multiplex PCR and none of the primers specific to caliciviruses were used in the reaction, as stressed earlier, a random amplification step was performed prior to biotin-labelling and that might have sensibly increased the number of targets. The presence of PEDV and coronavirus in two samples signifies the effective presence of coronavirus as these two viruses belong to the same family of *Coronaviridae* and they might share a high degree of sequence similarity.

Sample 25T was negative by multiplex PCR and positive by monoplex PCR for BVDV, the results showed a positive hybridisation to two positive probes, Bovine coronavirus and Bovine norovirus. The array was unable to detect BVDV in the samples, as it was not detected by multiplex PCR, which was initially the labelled material used in the hybridisation. This also indicated that random amplification did not impact the amplification of target concentration of BVDV

in this sample. Eventually it could be hypothesised that only a very low viral load was present in the sample.

All bovine clinical samples were screened by PCR prior to hybridisation, targeting pathogens by multiplex PCR that targeted not only viruses but also bacteria and parasites. It is thus a possibility that amplified targets by PCR were exclusively from bacteria and parasites. Yet again, the presence of only a very low viral load in the sample might also be a reason for the failed amplification of the viral targets.

Chapter 6. Design and Evaluation of Agilent Microarray

6. 1. Introduction

The present chapter follows the same approach as the previous chapter, specificity and sensitivity testing using known reference strains followed by its evaluation using porcine and bovine clinical samples.

The Agilent platform used in this study consists of a high density microarray slide that comprises four microarrays, with each microarray holding 44000 probes with appropriate controls.

A total number of 15993 probes were designed and spotted on the array in duplicate using 2612 sequences corresponding to viral, bacterial, parasite and host (bovine and porcine) genes. These probes include also all viral probes from the ArrayTube microarray. This array was thus far more comprehensive for initial laboratory assessment studies. A glass slide-based array would not be of any practical value, but served to evaluate the detection of multiple microbial species; however, the Alere array may form a practical platform for field studies in the future.

The specificity of the microarray was evaluated by specific amplifications, multiplex and monoplex PCR, either with reference strains or in clinical samples that were demonstrated to contain more than one pathogen. This aspect of the work was not designed to assess specificity in PCR-negative samples and therefore, array analyses were not performed on these samples, essentially for economic reasons.

The sensitivity of the microarray was assessed by applying different concentrations of the pathogen DNA to the array and identifying the detection limit of the arrays, it was then compared to PCR, qPCR and for bacteria colony count number.

Pathogens were finally identified in porcine and bovine faeces clinical samples, in which the presence was confirmed by monoplex and multiplex PCR. All hybridisation assays were carried out after specific amplification followed by a random amplification to perform the labelling.

6. 2. Material and Methods

6. 2. 1. Extraction using QIAamp® UCP Pathogen Mini Kit from broth

Bacterial DNA was extracted from overnight nutrient broth cultures by centrifuging 200 µl at 10,000 x g for 10 min and the supernatant discarded. The pellet was resuspended in 500 µl of lysis buffer ATL (with added DX reagent that helps reducing foaming of lysis buffer) and vortexed for 2 min at maximum speed; the tubes were then spun down. A volume of 400 µl of supernatant was mixed with 40 µl of proteinase K, vortexed for 10 sec and incubated at 56°C for 10 min. A volume of 200 µl of lysis buffer APL2 was added; the tube was vortexed and incubated at 70°C for 10 min. A total of 300 µl of absolute ethanol were added and 600 µl of the mixture subjected to filtration in a QIAamp UCP mini spin column placed in a 2 ml collection tube. The assembly was centrifuged at 6,000 x g for 1 min. Two washings of the spin column were performed with

600 µl of APW1 (both APW buffers supplied with the kit), the tubes were then centrifuged at 6,000 x g for 1 min, followed by adding 750 µl of APW2 and centrifuging at 20,000 x g for 3 min. To dry the column membrane, an additional centrifugation at full speed and incubation of the assembly on a thermomixer at 56°C for 3 min were performed. The column was placed in a new collection tube in both operations.

Elution was done by adding 60 µl of buffer AVE, supplied with the kit, to the spin column placed in a 1.5 ml microcentrifuge tube and incubation at room temperature for 1 min, followed by centrifugation at 20,000 x g for 1 min. Elution was repeated to increase the DNA yield by applying the eluted solution once again to the membrane. The eluate was then stored at -20°C until used.

6. 2. 2. Fluorescent labelling (Round C)

Labelling with Cy-3 and Cy-5 dyes (GE Healthcare Life Sciences - Buckinghamshire UK) was carried out using random amplification of PCR products that were subjected beforehand to round A and B amplification (Chapter 5- 5.2.4.1-b and 5.2.4.2). The reaction was performed in a 50 µl total volume, the master mix consisting of 29 µl of water, 2 µl of 50mM $MgCl_2$, 5 µl of 10x Mg-free buffer, 5 µl of dCTP labelling mix (1.2 mM dATP, 1.2 mM dTTP, 1.2 mM dGTP and 0.6 mM dCTP), 3 µl of Cy3-dCTP and/or Cy5-dCTP (GE Healthcate Life Sciences), 0.5 µl of 100 µM primer B and 0.5 µl of Taq polymerase. A volume of 5 µl of round B product was added and the mixture and subjected to 25 cycles of the same cycling conditions as in round B in the thermal cycler.

6. 2. 3. Hybridisation on Agilent slide

PCR products were random-amplified and labelled (5- 5.2.4.1-b and Chapter 6 – 6.2.3). Hybridisations were performed using an automated hybridisation station (Tecan HS Pro 400™). The software (HS Pro Control Manager) was programmed with the hybridisation conditions and bottles filled with the necessary volume of reagents (Prehybridisation buffer, Washing buffer 1 and Washing buffer 2) were connected appropriately in the station (Table 6.1). The slide was then assembled and locked in the hybridisation chamber. Hybridisation programme was then run.

6. 2. 3. 1. Sample preparation

a. Sample clean up

Samples were first cleaned up by applying 40 µl of round C labelled product to a Millipore column (10 kDa) (Microcon®), to which 40 µl of nuclease-free water were added and centrifuged for 15 min at 2400 x g. An additional 100 µl of nuclease-free water were added and the column was centrifuged at 2400 x g for 30 min. The sample was finally recovered by inverting the column in a new 1.5 ml microcentrifuge tube and pulsing briefly on a microfuge.

b. Hybridisation mix preparation

For each microarray, two samples were labelled separately with Cy3 and Cy5, then mixed together prior to hybridisation. A volume of 14 µl of each sample was mixed with 7 µl of 10 x blocking agent (Agilent) with a final volume of 35 µl, to which 35 µl of 2x GE hybridisation buffer were added and mixed by pipetting.

c. Programming hybridisation

Agilent microarray slide was placed with the barcode up in the hybridisation chamber of the hybridisation station. Hybridisation and washings were performed according to the programme in table 6.1.

Table 6.1. Hybridisation programme

Hybridisation steps	Temperature (°C) and duration (min/sec)
Pre-hybridisation	65°C for 2 min with soaking for 10 min
Sample injection	60 µl of sample injected by pipetting with the station set at 65°C with agitation
Hybridisation	65°C for 4h with agitation
Washing 1(Agilent)	23°C for 2 min 30 sec with soaking for 1 min 30 sec
Washing 2 (Agilent)	37°C for 2 min 30 sec with soaking for 1 min 30 sec
Slide drying	30°C for 2 min (under nitrogen at 30 Hg)

d. Slide scanning

Slides were scanned immediately with a GenePix® 4000B scanner (Molecular Devices – USA) with adjusted pixel resolution to 5 µm. The arrays were scanned using a dual laser system to scan two wavelengths simultaneously (532 nm and 635 nm). The scanner was equipped with GenePix Pro version software that captured images, generated output scanning files and results files. Data were exported for further analysis.

6. 2. 4. Specificity of Agilent microarray

A total of 12 individual reference strains of bacteria, viruses and parasites were used for the specificity testing; bacteria included *Salmonella* Typhimurium, *S.* Enteritidis, *E. coli* F4 and *E. coli* F5, *C. difficile, C. jejuni* and *B. pilosicoli*. Viruses included Porcine rotavirus A, TGEV, PEDV and PCV-2. The coccidian parasite *Eimeria acervulina* was included. All pathogens were amplified using primers

specific for the 16S rRNA gene for all bacterial strains, except for *E. coli* F4 and *E. coli* F5 for which the F4ac *faeG* and K99 fimbrial subunit genes were amplified. The VP7 gene was amplified for Porcine rotavirus, ORF1b for TGEV, the membrane-nucleocapsid-envelope for PEDV, the replicase gene for PCV2 and 18S rRNA for *E. acervulina*.

To assess the specificity of the array when multiple pathogens are present, two multiplex PCR reactions were performed as described for AT in Chapter 5 - 5.2.6.

In addition, bovine and porcine genomic DNA was used as a template in a PCR reaction to amplify the mitochondrial cytochrome b gene.

PCR products were then randomly amplified (Chapter 5- 5.2.4.1 and 5.2.4.2). Labelling with Cyanine Cy3 and Cy5 was done so that strains from different kingdoms were labelled to be easily differentiated from each other (Chapter 6- 6.2.3). Labelled products were cleaned up, then pooled together according to table 6.2 and in each experiment, four samples were hybridised, each in a separate microarray, onto the slide.

Table 6.2. Samples hybridised on the Agilent array

Slide	Samples	Labelled with Cy3	Labelled with Cy5
Slide 1	Sample 1	*Salmonella* Typhimurium	Porcine rotavirus A
	Sample 2	*Clostridium difficile*	TGEV
	Sample 3	*Salmonella* Enteritidis	PEDV
	Sample 4	*Campylobacter jejuni*	PCV2
Slide 2	Sample 1	Multiplex 1	Multiplex 2
	Sample 2	Bovine cytochrome b	Porcine cytochrome b
	Sample 3	*E. coli* F4	*Brachyspira pilosicoli*
	Sample 4	*E. coli* F5	*Eimeria acervulina*

6. 2. 5. Sensitivity of Agilent microarray

The sensitivity testing of the Agilent array was carried out using one virus (PCV-2) and a bacterium (*E. coli* F5). Viral DNA was two-fold serially diluted and subjected to a PCR reaction for the detection of a 543 bp fragment by 1% gel electrophoresis. *E. coli* F5 was cultivated in nutrient broth overnight at 37°C and serially diluted 10-fold. Bacterial DNA was extracted from 20 µl of pelleted bacteria from dilutions 10^{-3} to 10^{-8} of nutrient broth culture using QIAamp UCP Pathogen Mini Kit (Qiagen) (see 6.2.1). Concurrently, 200 mg of faeces from apparently healthy cows were spiked with pelleted bacteria from 20 µl of each dilution after being centrifuged at 10,000 x g for 10 min. The DNA was then extracted from spiked faeces with dilutions 10^{-1} (4.17×10^{11} copy number) to 10^{-6} (4.17×10^{6} copy number) using QIAamp DNA Stool Mini Kit (Qiagen) (Chapter 4- 4.2.4). Additionally, a non-spiked faecal sample was included. All dilutions were plated out in triplicate on MacConkey agar and the CFU (Colony Forming Unit) counted after 24h incubation at 37°C (Fig. 6.1). Specific PCR reactions were carried out with DNA extracted from dilutions 10^{-3} (4.17×10^{9}

copy number) to 10^{-6} (4.17 x 10^6 copy number) and spiked faeces with dilution 10^{-1} (4.17 x 10^{11} copy number) to 10^{-6} (4.17 x 10^6 copy number). The primers used were to amplify the K99 fimbrial subunit gene. PCR products of 1120 bp were visualised on 1% agarose gel electrophoresis stained for fluorescence with Nancy-520 (10%).

PCR products obtained from gene amplifications of PCV-2 (2ng (2.065 x 10^9 copy number), 1ng (1.032 x 10^9 copy number), 0.5ng (5.164 x 10^8 copy number), 0.25ng (2.582 x 10^8 copy number) and *E. coli* F5 (spiked faeces with dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, (respectively 4.17 x 10^{10}, 4.17 x 10^9, 2.08 x 10^8, 4.17 x 10^7 copy number), were first randomly amplified (Round A and B – Chapter 5 – 5.2.4.1-b and 5.2.4.2), labelled respectively with Cy-5 and Cy-3 fluorescent dyes (Round C - Chapter 6– 6.2.3) and simultaneously hybridised to the Agilent array.

6. 2. 5. 1. Quantitative real time PCR

To assess the detection sensitivity of the Agilent array, Real-time PCR was performed for bovine faeces spiked with six dilutions (-1 to -6, 4.17 x 10^{11} to 4.17 x 10^6 copy number) of *E. coli* F5. A negative control and a non-spiked sample were introduced in the assay.

The reaction was carried out in triplicates in a total volume of 20 µl. The master mix comprised 7 µl of water, 10 µl of probe master 2x concentrated (Roche), 1 µl of primer mix (5 µM), 1 µl of probe mix (2 µM) (Table 6.3) and 1 µl of template. The amplification was performed in a sealed 96 multiwell plate (Roche) in a qPCR LightCycler 480 (Roche). Cycling conditions were as follows:

initial denaturation for 10 min at 95°C, 57 cycles of 95°C for 10 sec, 57°C for 30 sec and 72°C for 2 sec. The probe (1 OD- Purification: HPLC- Sigma Aldrich– Life Science) was dual-labelled with a reporter at the 5' end (6-FAM™) and a quencher at the 3' end (TAMRA™). Results were obtained from the LightCycler 480 software 1.5.0.

Table 6.3. Designed *E. coli* F5 qPCR primers and probes

Sequence	Primer / Probe name	Primer / Probe sequence
Forward primer	qPCR_E_coli_F5_F	GTGTCAGTGGAGATGGGATTAC
Reverse primer	qPCR_E_coli_F5_R	CCGCATATTTTATACCGCCAAG
Probe	E_coli_F5_Probe	ATGCCATTGGGAAAACGCCATGAC

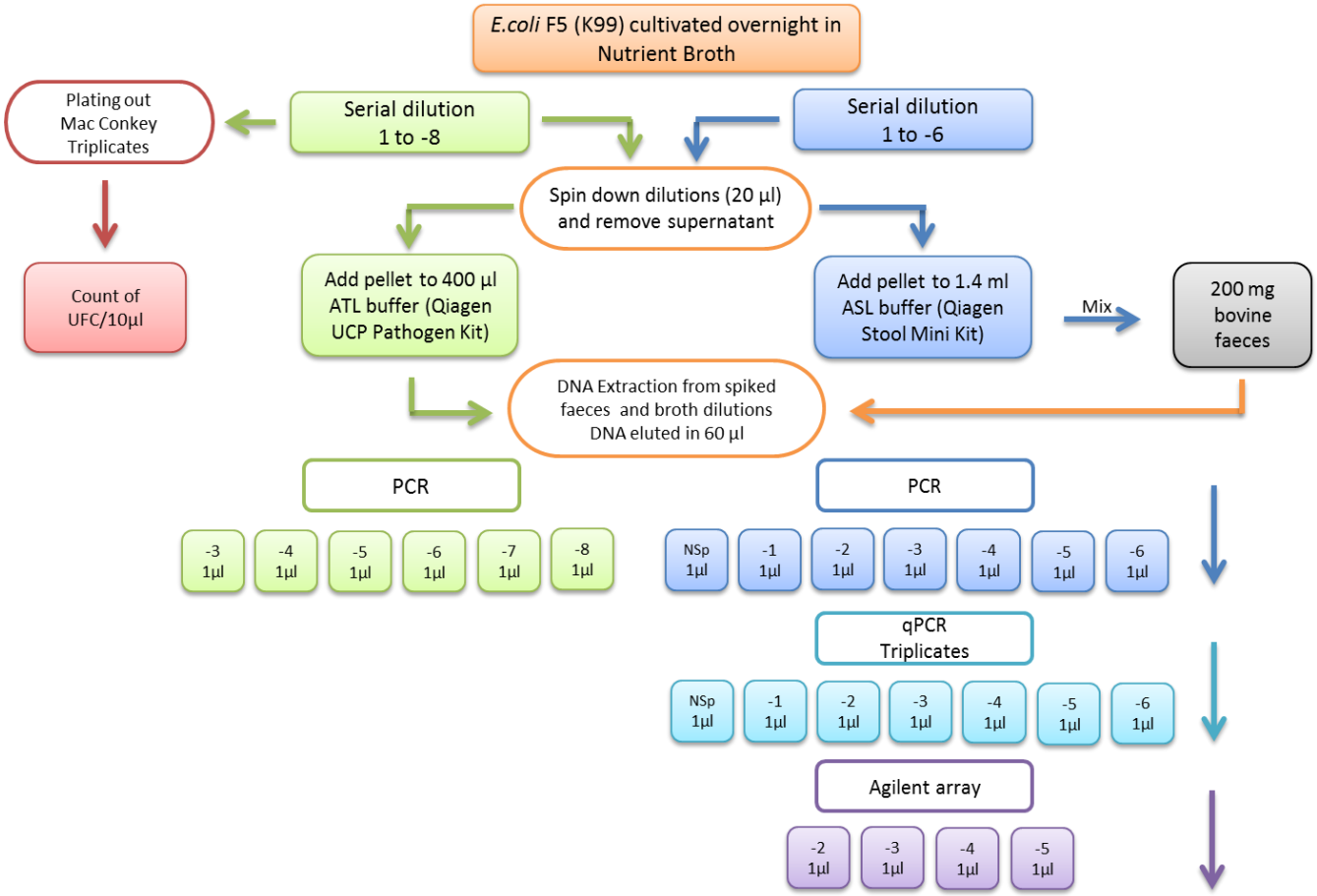


Figure 6.1. Illustration of the workflow for the sensitivity test on Agilent microarray

NSp: Non-spiked

6. 2. 6. Hybridisation of clinical samples to Agilent platform slides

A total of eight porcine and bovine (4 each) faecal samples were hybridised to an Agilent slide. Porcine samples consisted of samples 8, C, 4B and 7B cited in table 5.2. Bovine samples (1/3, 20T, 1 and 55) were positive by multiplex PCR showing 1 to 3 bands on electrophoresis gel. It was not possible to identify the pathogens that were present in these samples because the amplified PCR products have very similar molecular weights; however the size of all amplicons was higher than 1000 bp. Only positive clinical samples were subjected to array hybridisation, whereas all the samples were screened using monoplex and multiplex PCR.

6. 2. 7. Data analysis

The Agilent raw data results were normalised using quantile normalisation (Hicks and Irizarry, 2014) with a microarray data analysis software, GeneSpring 13.0 (Agilent). This was to reduce variation between arrays due to technical conditions during the experiment that are difficult to control. Also prior to normalisation, the software automatically averaged signal intensities of duplicated probes, as well as a $\log_2$ transformation.

The cut-off signal was determined in the same manner as for the Alere platform. For bovine and porcine clinical samples, all probes originating from ribosomal sequences were removed from the output data.

To determine which software generated the most specific probes, the number of positive probes per software for each reference strain that was correctly

identified were compared using Fisher's test. Equally, the number of false positive probes were compared using the same test.

In this chapter and in all figures, the number of plotted spot intensities per array were dependent on the total number of probes per organism on the array, but also on the extent of cross-hybridisation, as it was meant to show probes with intensities that were close to the cut-off values.

6. 3. Results

6. 3. 1. Specificity of Agilent platform

6. 3. 1. 1. Specificity for unique species/strain hybridisation

A total of 16 hybridisation assays were carried out to interrogate the specificity of the Agilent platform. Four species of viruses (Porcine rotavirus A, TGEV, PEDV and PCV2), seven species/strains of bacteria (*S.* Typhimurium, *S.* Enteritidis, *C. difficile*, *C. jejuni*, *E. coli* F4 (K88), *E. coli* F5 (K99) and *B. pilosicoli*), and one species of parasite (*E. acervulina*) were tested. Additionally, host (porcine and bovine) DNA cytochrome b were fluorescently labelled and hybridised to the array.

Porcine rotavirus A array hybridisation showed that only seven porcine rotavirus probes out of 411 hybridised to target (Fig. 6.2 - A), and which were above the cut–off of 6.75, with the highest signal intensities varying between 6.81 and 8.07. This, in general contrasts with the behaviour of the Alere array where P. rotavirus performed poorly. More than half the probes were designed with eArray (57.14%, n=4) and 42.82% (n=3) with UPS.

Fig. 6.2 – B, illustrates successful identification of TGEV on the array. Among probes with highest signal intensities (4.78 to 8.02), a total of 129 out of 151 probes on the array hybridised with the TGEV genome; however, 33 were above the signal cut-off (6.17). Host genome hybridised with cytochrome b probes represented by a set of 35 probes and their signals varied from 2.87 to 5.85. The presence of host DNA in the sample might have resulted from DNA

carry-over during the extraction process. Among all TGEV positive probes (n= 33), 45.45% (n= 15) were designed with UPS, 36.36% (n=12) with Picky, 18.18% (n=6) with eArray. None of the probes designed with GoArray was above the cut-off.

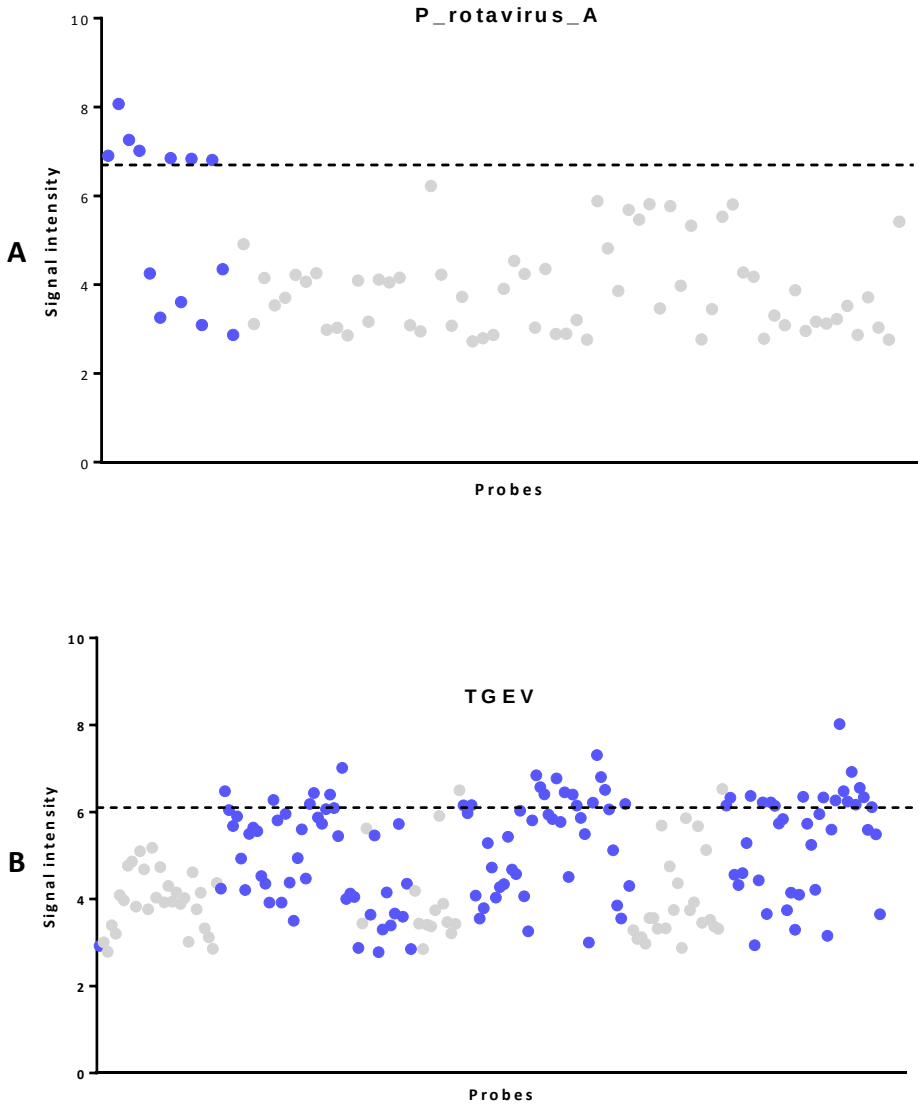


Figure 6.2. Normalised signal intensities following hybridisation of Cy5-labelled DNA of P. rotavirus A (A) and TGEV (B) on Agilent platform

Probes specific to the pathogen are displayed in blue, grey spots are cross-hybridised probes. Signal cut-off of 6.75 for P. rotavirus and 6.17 for TGEV is represented by a dashed horizontal line. Seven and 33 probes have signals higher than the cut-off respectively for P. rotavirus and TGEV. Only two probes (Teschovirus polyprotein and integron integrase genes) cross-hybridised with TGEV target with signals of 6.53 and 6.49 respectively.

The PEDV hybridisation result (Fig. 6.3 – A) showed a more uniform pattern of positive probes corresponding to PEDV, with highest signal intensities starting at 7.95. Of a total of 212 probes specific to PEDV, 178 PEDV probes were above the intensity cut-off limit of 3.86 and only one probe was specific to *C. difficile* toxin B (*tcdB*), below the cut-off limit, with a signal intensities of 3.19,.

The highest proportion of positive probes was represented by UPS- and eArray-designed probes with 46.63% (n= 83) and 28.65% (n= 51), respectively, then for GoArray and Picky at 15.17% (n= 27) and 9.55% (n=17).

PCV2 hybridisation results (Fig. 6.3 –B) showed 241 PCV2/1 positive probes with the highest signals ranging from 7.97 to 3.75. Among these positive probes (with signals higher than 4.48), only two probes (U_L_int_T3SS_16U and P_S_new_par_06, designed from type 3 secretion system and CMY-2 family class C beta-lactamase genes) cross-hybridised with PCV2 with a signal intensity of 4.72 and 4.50 respectively. Out of 129 positive PCV1/2 probes, 44.96% (n= 58) were designed by UPS, 31.78 % (n= 41) by eArray, 17.05% (n= 22) by Picky and (n= 8) 6.20% by GoArray software.

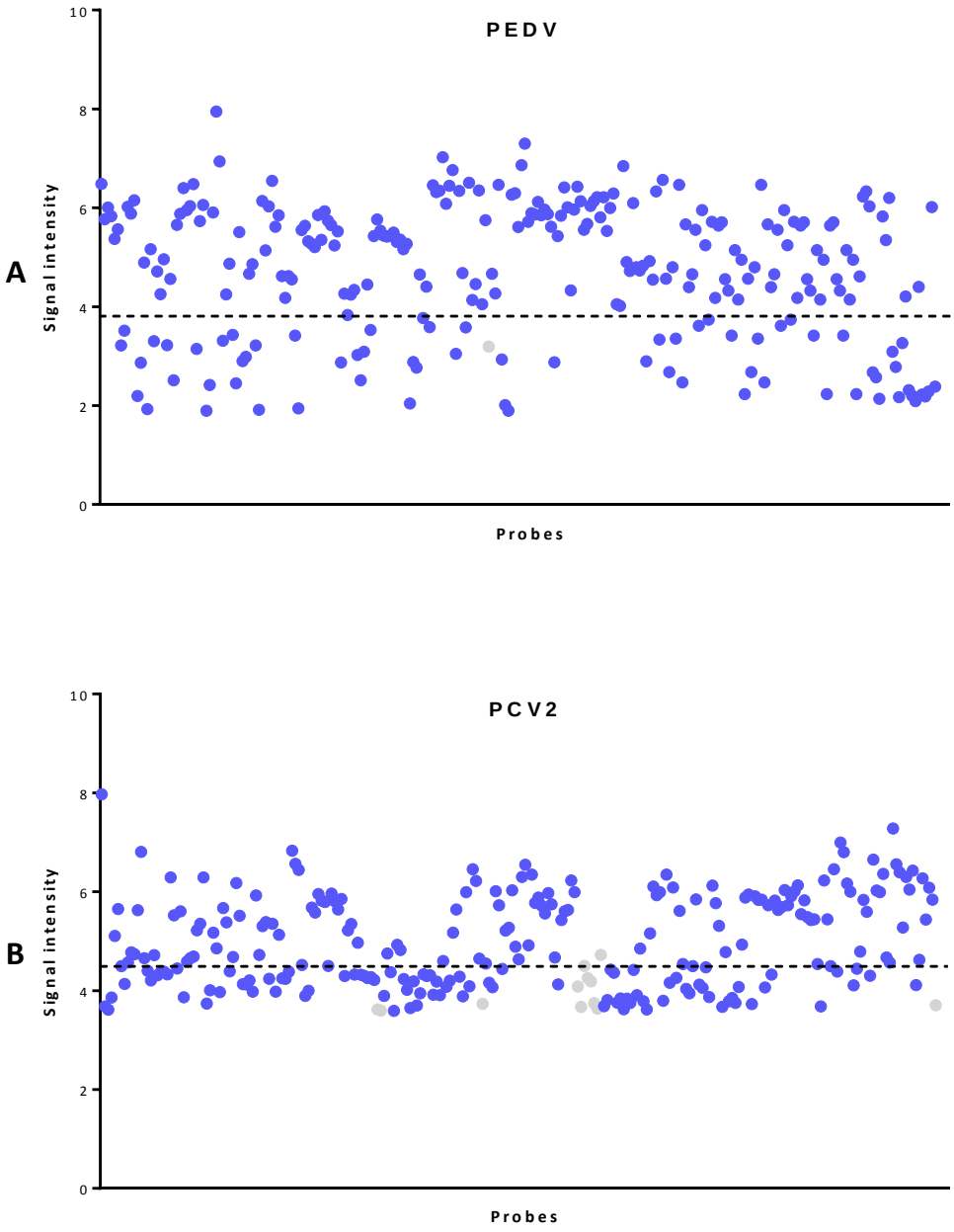


Figure 6.3. Normalised signal intensities following hybridisation of Cy5-labelled DNA of PEDV (A) and PCV2 (B) hybridised into Agilent platform

Probes specific to the pathogen are displayed in blue. Grey spots are cross-hybridised probes. Signal cut-off of 3.86 for PEDV and 4.48 for PCV2 is represented by a dashed horizontal line. A total of 178 PEDV probes and 91 PCV1/2 probes had signal higher than their respective cut-off.

When DNA from *S.* Typhimurium was hybridised on the array, a high level of cross-hybridisation was observed with targets in *Salmonella* other than *S.* Typhimurium (Fig. 6.4). In effect, the amplified target was specifically the 16S rRNA, which led us to decide not to remoive the probes designed from this ribosomal gene. Only three positive probes were specific to *S.* Typhimurium, while 15 positive probes were specific to *S.* Enteritidis. Other probes (n=4) designed from other *Salmonella* serovars such as *S*. Choleraesuis and *S.* Dublin also showed positive hybridisation with *S.* Typhimurium. It is important to point out that all positive probes were designed from the 16S rRNA region of *Salmonella* genome. Three *Yersinia enterocolitica* probes cross hybridised with *S.* Typhimurium, these also being sequences from the 16S rRNA gene.

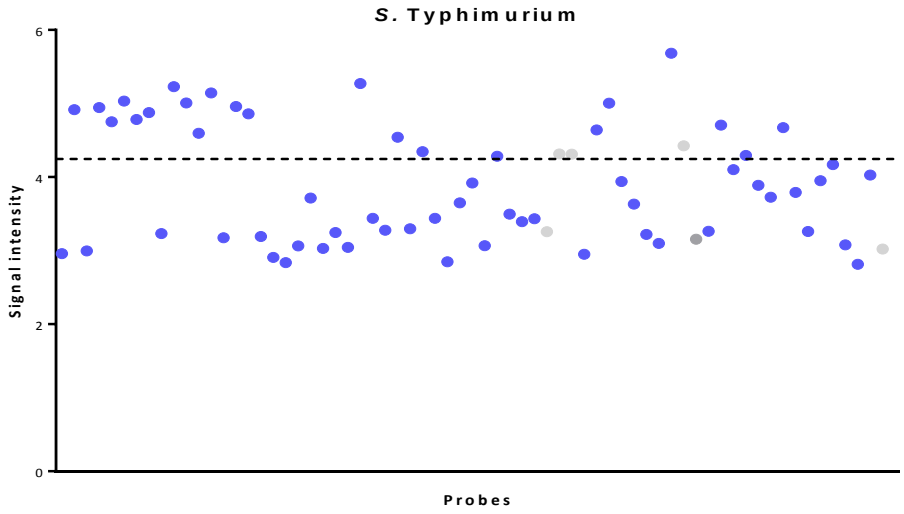


Figure 6.4. Normalised signal intensities following hybridisation of Cy3-labelled DNA of *S.* Typhimurium on Agilent platform.

Blue spot: 16S rRNA of all *Salmonella* serotypes including *S.* Typhimurium, *S.* Enteritidis, *S.* spp, *S.* Dublin, *S.* Choleraesuis, and *S.* Newport, Light grey : *Y. enterocolitica*, Dark grey : *C. jejun*i. Signal cut-off of 4.23 is represented by a dashed horizontal line. The figure shows 22 probes (blue spots) specific to *Salmonella* spp. and three cross-reating *Y. enterocolitica* 16S probes (light grey spot).

As for hybridisation with *S.* Enteritidis DNA (Fig. 6.5), 12 probes among 13 were specific to *S.* Enteritidis, only one Choleraesuis serovar probe was represented. Again this probe was specific to the 16S rRNA of *S.* Enteritidis. The signal cut-off value was 4.69.

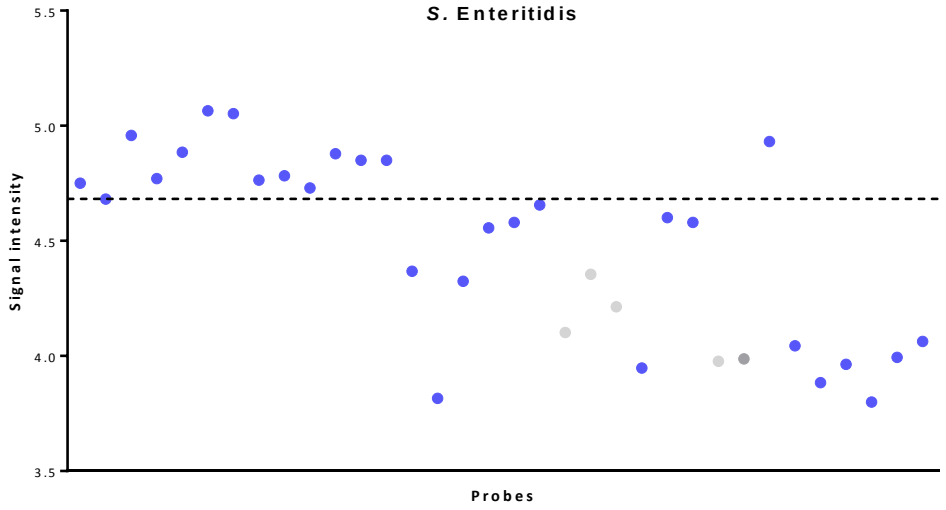


Figure 6.5. Normalised signal intensities following hybridisation of Cy3-labelled DNA of *S.* Enteritidis on Agilent platform

Blue spot: 16S rRNA of all *Salmonella* serotypes including *S.* Enteritidis, *S.* Typhimurium, *S.* spp, *S.* Dublin and *S.* Choleraesuis. Light grey spot: *Y. enterocolitica,* Dark grey: *C. jejuni.* Signal cut-off of 4.69 is represented by a dashed horizontal line. Except one specific probe to *S.* Choleraesuis, the remaining 12 probes were designed from Enteritidis 16S rRNA and have signals higher than the cut-off.

Among 35 specific probes to *S.* Typhimurium and Enteritidis with signals higher than their respective cut-offs, 74.29% (n=26) were designed using eArray and only 11.43% (n=4), 8.57% (n=3) and 5.71% (n=2) were computed with UPS, Picky and GoArray respectively.

Hybridisation signal intensities of *C. difficile* (Fig. 6.6) showed that 50% of probes with signal intensities higher than the cut-off (4.78) were specific to *C. difficile*. Eleven (50%) probes were positive to *C. perfringens*, although probes and targets all originated from the 16S rRNA. In terms of probe design software performance, eArray scored the highest number of positive probes with 13 out of 22 (59.09%), GoArray and UPS recorded 22.73% (n=5) and 18.18% (n=4) respectively.

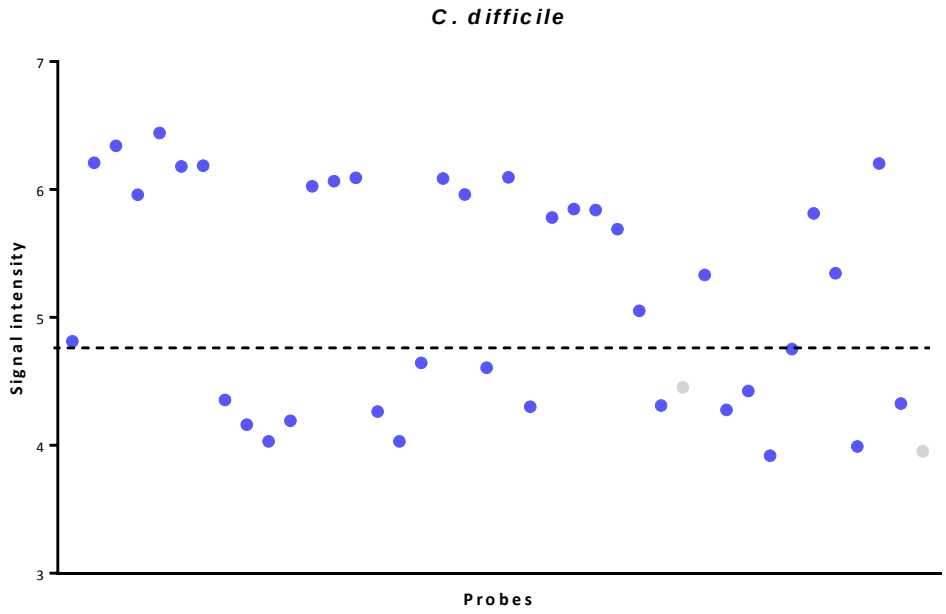


Figure 6.6. Normalised signal intensities following hybridisation of Cy3-labelled DNA of *C. difficile* hybridised into Agilent platform

Blue spot: 16S rRNA of *C. difficile* and *C. perfringens*, Light grey spot*: L. intracellularis.* Signal cut-off of 4.78 is represented by a dashed horizontal line. The 22 probes with signals above the cut-off were in totality representative of the 16S rRNA of *C. difficile/perfringens.*

When *C. jejuni* was hybridised to Agilent microarray (Fig. 6.7), 71 *C. jejuni* probes were positive. Probes corresponding to *C. coli* hybridised with *C. jejuni* target, yet again because both probes and targets were from the 16S -23S intergenic spacer. Minimal cross-hybridisation with other species was observed with *S.* Dublin, *S.* Enteritidis, *S.* Typhimurium, *C. difficile*, *C. perfringens* and *L. intracellularis*, the probes of these species corresponded to the 16S rRNA genes.

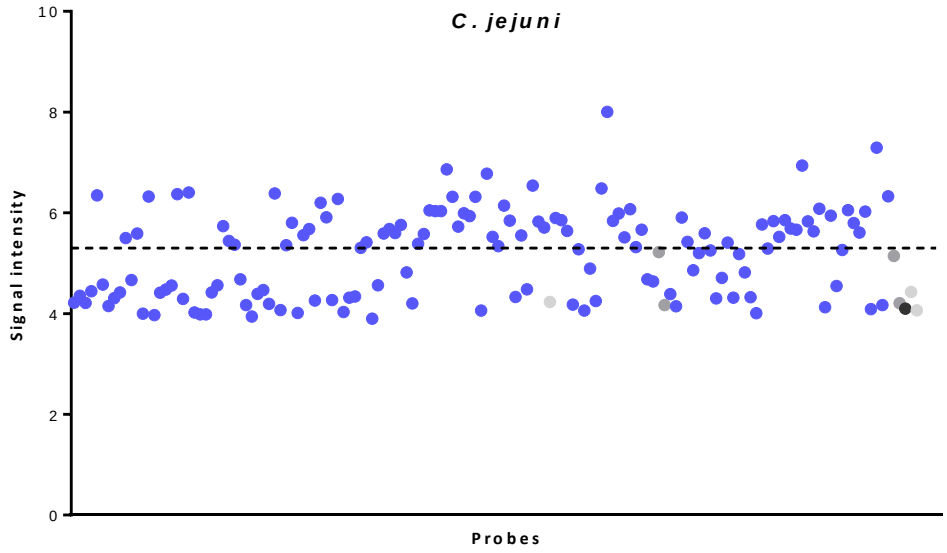


Figure 6.7. Normalised signal intensities following hybridisation of Cy3-labelled DNA *of C. jejuni* hybridised into Agilent platform

Blue spot: 16S rRNA of *C. jejuni* and *C. coli*. Light grey spot: *Salmonella* spp., Dark grey: *Clostridium* spp., Black spot: *Lawsonia intracellularis*. A total of 71 specific probes to the ITS of *C. jejuni/coli* are above the signal cut-off of 5.36, which is represented by a dashed horizontal line.

Bovine and porcine cytochrome b genes were hybridised simultaneously to the same array (using two different dyes, Cy3 for bovine cytochrome and Cy5 for porcine cytochrome) to assess their specificity and to evaluate the extent of cross hybridisation of the host genes with pathogen probes. The results (Fig. 6.8) showed that among a total number of 64 probes of bovine cytochrome b on the array, 31 were above the 8.54 signal intensity cut-off, with a range of relatively high signals between 8.54 and 9.38. Among these probes 87.10% (n= 27) were designed by eArray and 12.90% (n=4) by UPS. None of the probes designed by Picky or GoArray, showed a signal intensity higher than the cut-off. The bovine cytochrome b gene cross-reacted with one porcine cytochrome b probe, one bovine enterovirus probe and one Campylobacter coli probe.

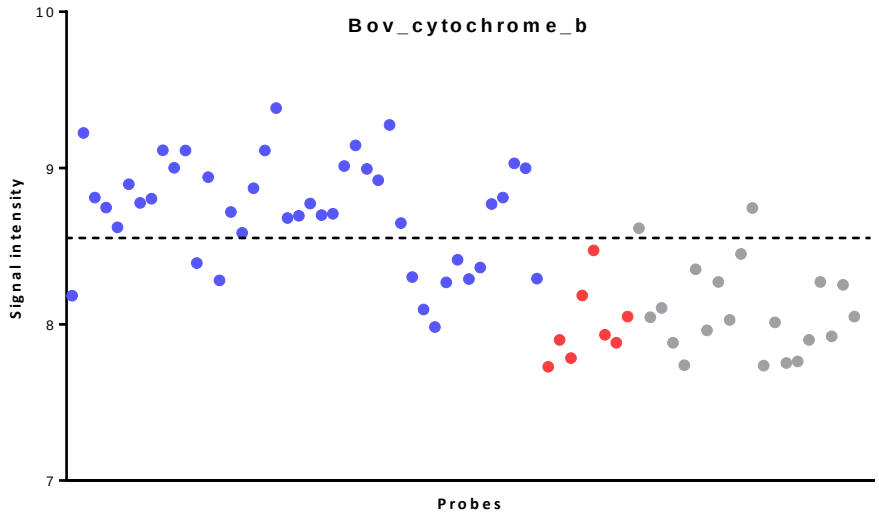


Figure 6.8. Normalised signal intensities following hybridisation of Cy3-labelled DNA of bovine cytochrome b gene amplicon

Blue spot: Bovine cytochrome b, Red spot: Porcine cytochrome b, Grey spots: cross-hybridising probes. Signal cut-off of 8.54 is represented by a dashed horizontal line. Only eight porcine cytochrome b probes reacted with bovine cytochrome b but they remained lower than the cut-off. Two viral and bacterial probes registred intensities higher than cut-off.

A lower proportion of positive probes was specific to porcine cytochrome b (Fig. 6.9), only 11 were above the cut-off of 8.66 (althought 42 out of 51 hybridised to the array). Positive specific probes to porcine cytochrome b that were designed by eArray scored 45.45% (n=5) while other probes counted for 36.36% (n= 4) , 18.18% (n= 2) and 0% respectively for GoArray, UPS and Picky software. Also, only two bovine cytochrome b probes cross-hybridised with the porcine host DNA and no pathogen-specific probes hybridised with the porcine DNA above the cut-off.

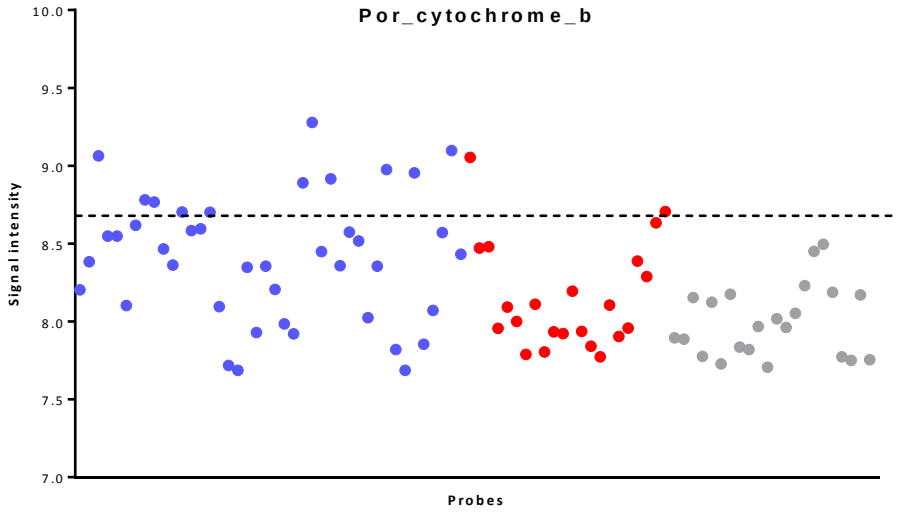


Figure 6.9. Normalised signal intensities following hybridisation of Cy5-labelled DNA of porcine cytochrome b gene amplicon

Blue spot: Porcine cytochrome b, Red spot: Bovine cytochrome b, Grey spots: Cross-hybridising probes. Signal cut-off of 8.66 is represented by a dashed horizontal line. Two bovine cytochrome b with signals of 9.05 and 8.70 were above the cut-off limit.

Nucleic acid from the *E. coli* F4 and *E. coli* F5 reference strains were also hybridised, but in separate arrays to prevent cross-reactions.

Hybridisation of *E. coli* F4 to the array (Fig. 6.10) showed that a total of 14 probes were positive for the F4 gene (Blue spots), 12 of which were above the cut-off of 7.57. There were 136 positive probes (Red spots) corresponding to 19 virulence genes and 20 probes which were virulence-specific and above the cut-off. These comprised heat stable enterotoxin 1 (*astA*), labile toxins a/b (*LTa* and *LTb*), cadaverine/lysine antiporter (*cadB and cadC*), usher /outer membrane usher gene (*aggR*) and transposases. Twenty probes showed the presence of antibiotic resistance genes (Green spots) and only one probe representing class II integron multidrug resistance had a signal of 7.92, higher than the cut-off. It is likely that these virulence and antibiotic genes have a plasmid origin. In fact, the targeted genes (F4 and F5) are located on a plasmid, alongside enterotoxins and antibiotic resistance genes.

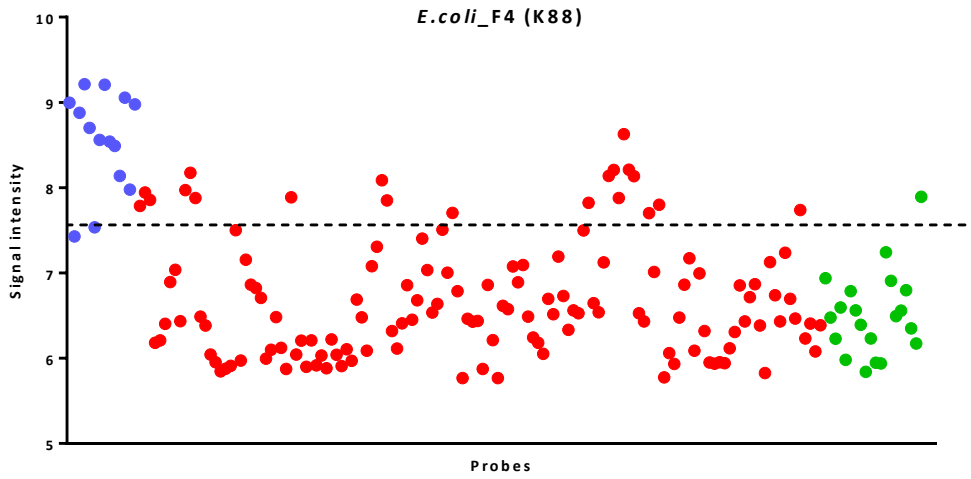


Figure 6.10. Normalised signal intensities following hybridisation of Cy3-labelled DNA of F4 gene of *E. coli* F4 (K88) to Agilent platform

Blue spot: *E. coli* F4, Red spot: Virulence genes, Green spot: Antibiotic resistance genes. Signal cut-off of 7.57 is represented by a dashed horizontal line. Twelve probes were specific to ETEC F4 (Blue spot) with relatively high signals of 7.70-9.21. Red spots represent virulence genes including heat stable enterotoxin (*astA*), labile toxin a and b (*LTa/b*), lysine/cadaverine antiporter (*cadB* and *cadC*) and transcriptional activator of aggregative adherence (*aggR*).

As for *E. coli* F5, Figure 6.11 shows that 23 positive probes) were specific to F5 gene (Blue spots), nine of which were above the cut-off of 8.48. In addition, three virulence genes (Red spots above the cut-off line) showed relatively high signals (8.86 to 9.00), they included plasmid IS4 family transposase and RNA I and II genes.

Virulence genes or genes playing a role in the virulence mechanism of *E. coli* have been identified through 58 probes (Red spots), in addition to 30 genes specific to antibiotic resistance (Green spots). These sets of probes correspond to genes that may frequently be carried by plasmids and have been amplified during the labelling of the F5 gene amplicon that involved a random amplification.

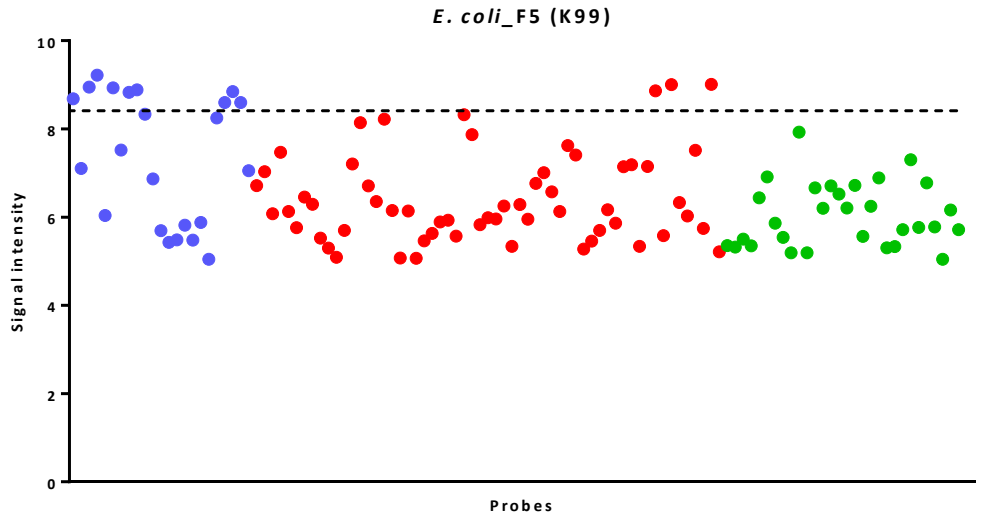


Figure 6.11. Normalised signal intensities following hybridisation of Cy3-labelled DNA of F5 gene of *E. coli* F5 (K99) to Agilent platform

Blue spot: *E. coli* F5, Red spot: Virulence genes, Green spot: Antibiotic resistance genes. Nine ETEC F5 probes (blue spots) and three virulence probes (Red spots) showed signals higher than the cut-off of 8.48 is represented by a dashed horizontal line. ETEC F5-specific probes had a signal of 5.04 to 9.22.

Among the probes that correctly identified *E. coli* F5 (n=9) and F4 (n=12) genes, 33.33% (n= 3) and 41.67% (n= 5) were respectively designed by UPS software. Picky-designed probes were 33.33% for F5 and F4 (n= 3 and n= 4 respectively). Probes with eArray were represented by 22.22% (n= 2) for F5 probes and 25% (n= 3) for F4 probes, while GoArray probes represented only 11.11% (n= 1) and 0% of the total fimbrial F5 and F4 probes, respectively.

The PCR product of *B. pilosicoli* 16S rRNA bound to 147 probes derived from *B. pilosicoli, B. hyodysenteriae* and *Brachyspira* spp. (Fig. 6.12). All probes were specific to *Brachyspira* 16S rRNA (Blue spots), which explains the positivity of *B. hyodysenteriae* probes. Only two probes represented by *C. jejuni* cross-hybridised with *B. pilosicoli* (Grey spots), however with signal intensity below the cut-off of 5.53.

A high proportion of probes specific to *Brachyspira* were eArray probes (63.31%, n= 96), GoArray probes recorded 29.93% (n= 44) of the total number of probes. The percentage of specific probes to *Brachyspira* spp. 16S with signals higher than the cut-off counted only 3.40 (n=5) and 1.36 (n=2) respectively for UPS and Picky.

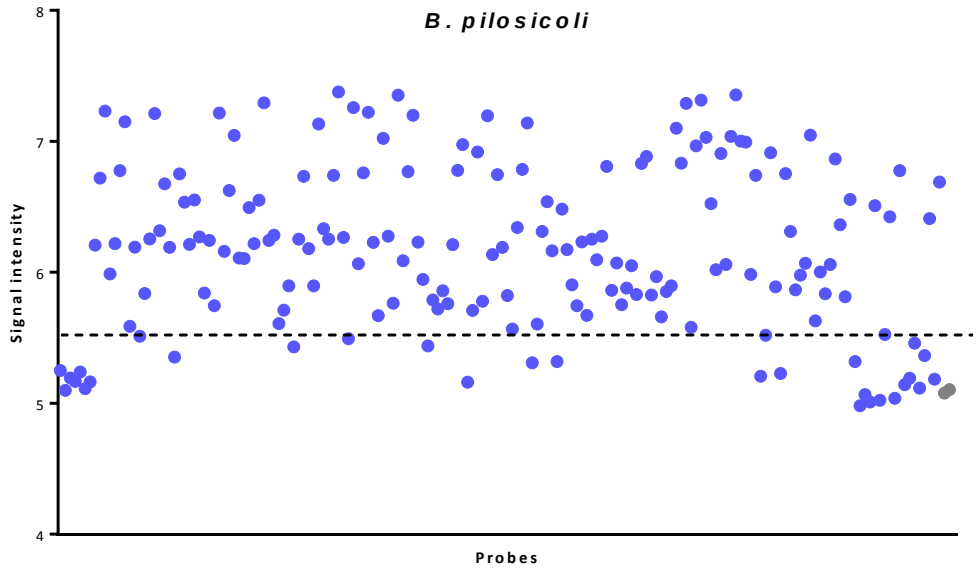


Figure 6.12. Normalised signal intensities following hybridisation of Cy5-labelled DNA of 16S rRNA of *Brachyspira pilosicoli* to Agilent platform

Blue spot: *B. pilosicoli, B. hyodysenteriae* and *Brachyspira* spp., Grey spots: *Campylobacter jejuni*. The signal cut-off of 5.53 is represented by a dashed horizontal line. A total of 147 probes were specific to *Brachyspira* spp. 16S rRNA, with signals varying between 5.56 and 7.37. Only two *C. jejuni* probes cross-hybridised with the target.

Hybridisation of *E. acervulina* 18S rRNA on the Agilent microarray (Fig. 6.13) showed a relatively high number of positive specific probes (n= 45), with signals above the signal cut-off of 7.66. The targeted gene was 18S rRNA, which confronted us with the same issue of bacterial 16S rRNA. As 18S rRNA is a highly conserved sequence among eukaryotic organisms, to counteract its low specificity, all probes corresponding to the parasite 18S rRNA as well as 16S rRNA were removed from the data. Only one bacterial probe cross-reacted with target DNA (EAEC_vagC_03) with a signal of 7.75.

Positive probes designed with eArray and UPS software were common among specific probes to the 18S rRNA, 40% (n= 18) and 35.56% (n= 16), respectively. GoArray and Picky probes resulted in 15.56% (n= 7) and 8.89% (n= 4).

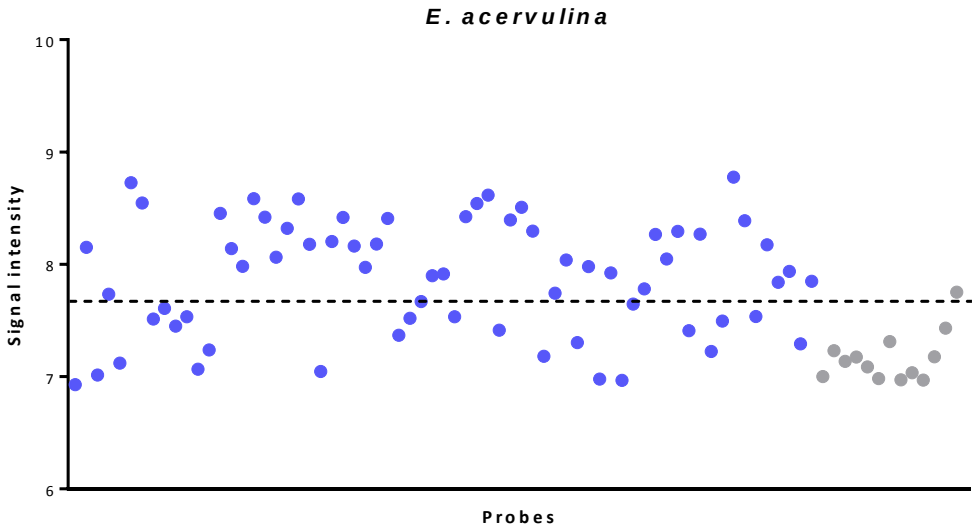


Figure 6.13. Normalised signal intensities following hybridisation of Cy5-labelled DNA of 18S rRNA of *Eimeria acervulina* to Agilent platform

Blue spot: *Eimeria* spp. 18S rRNA, Grey spot: Cross-hybridising probes. The signal cut-off of 7.66 is represented by a dashed horizontal line. One probe specific to *E.coli* showed a signal higher than the cut-off (grey spot above the dashed line). A total of 45 probes were specific to *Eimeria* 18S rRNA with signal varying from 7.66 to 8.77.

6. 3. 1. 2. Specificity of multiple species/strains hybridisation

Multiplex_1 and multiplex_2 were two sets of five pathogens each multiplexed and hybridised to the Agilent platform. Figure 6.14 illustrates the results of hybridisation of amplicons from the five pathogens in multiplex 1 (*S*. Typhimurium, *S.* Enteritidis, *C. difficile*, PCV2 and TGEV) to the array. For *Salmonella*, the target was the 16S rRNA gene with which all *Salmonella* serovars (Newport, Choleraesuis and Dublin), hybridised. Equally, the 16S rRNA of *Clostridium* reacted with both *C. difficile* and *C. perfringens*. This led to representing all *Salmonella* serovars and *Clostridium* species concomitantly in Fig. 6.14. Results revealed a predominance of *Clostridum* and PCV with probes showing the highest signals, probably due to the higher copy number of these pathogens in the sample. A total of 212 probes higher than the cut-off (3.45) hybridised to the array. A relatively low number of *Salmonella* and TGEV probes were positive, again possibly caused by a reduced amount of targets in the sample. Few non-specific hybridisation with probes representing other pathogens with signals starting from 2.38 to 3.49 were recorded, they include *Eimeria*, Coronaviruses and host cytochrome b.

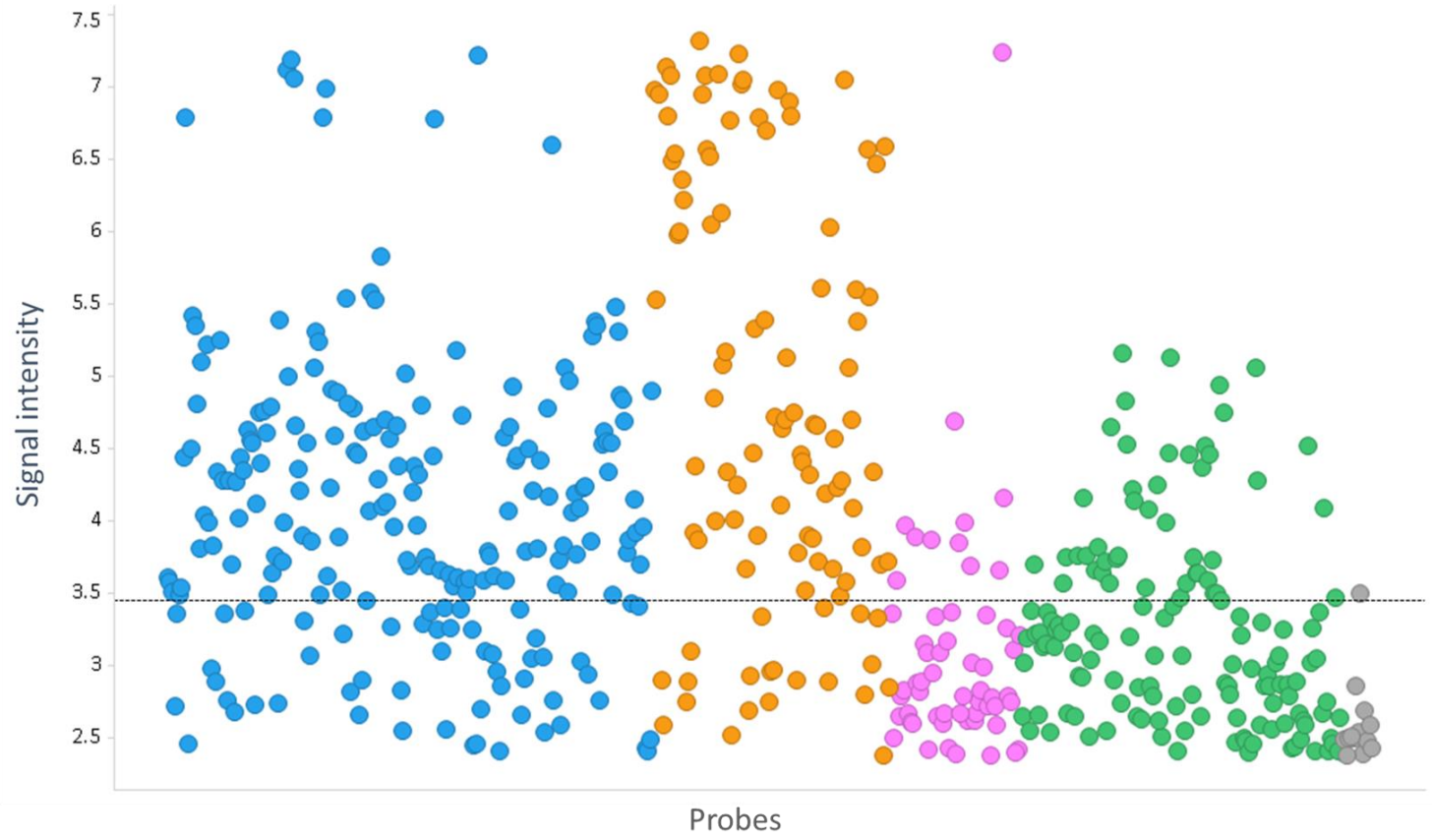


Figure 6.14. Normalised signal intensities following hybridisation of labelled DNA from five pathogens to Agilent platform (multiplex PCR- 1st set).

Blue spot: PCV1/2, Orange spot: *C. difficile/ C. perfringens*, Pink spot: TGEV, Green spots: All salmonella serovars, Grey spot: Cross-hybridised probes from other pathogens– Scatter plot was generated with TIBCO® Spotfire®.

A high number of positive PCV and *Clostridium* probes have hybridised (71 and 84 respectively) with high signals. *Salmonella* counted 46 probes and TGEV 11 probes with signals above the cut-off of 3.45, represented by a horizontal dashed line.

Table 6.4 demonstrates the number of probes detected per pathogen and software. Among a total of 212 signal intensities higher than the cut-off, 37.74% (n=80) and 34.43% (n=73) were identified by eArray and UPS respectively, then 17.92% (n=39) and 9.91% (n=21) of positive probes were designed by GoArray and Picky, respectively. In this multiplex hybridisation, only 13 probes cross-reacted with non-targets.

Table 6.4. Number of detected pathogens per software in multiplex_1

Software	PCV	TGEV	*C. diff./ C.perf.*	*Salm*.	Total
eArray	21	2	35	22	80
UPS	32	5	33	3	73
Picky	12	4	3	2	21
GoArray	6	0	13	19	39
Total	71	11	84	46	**212**

C. diff.: *Clostridium difficile*, *C. perf*.: *Clostridium perfringens*, *Salm*.: *Salmonella*

As for the Multiplex_2 hybridisation results, the multiplex reaction targeted among others, the 16S rRNA of *B. pilosicoli* and *B. hyodysenteriae* and the 16S rRNA of *C. jejuni* and *C. coli*. This led to taking into consideration all signal intensity results for these four species. The results (Fig. 6.15) showed clear detection of PEDV and *Brachyspira* species with corresponding probes exhibiting the highest signal intensities. Only seven rotavirus probes hybridied to the target. The majority of *C. jejuni* and *Eimeria* probes showed low signal intensities compared to the other species. The total number of hybridised probes with signals above the cut-off of 3.21 counted 488. The targets of the

multiplex cross-hybridised with 11 probes that were specific to Picornaviruses, Pestiviruses and Shiga-toxin *E. coli*.

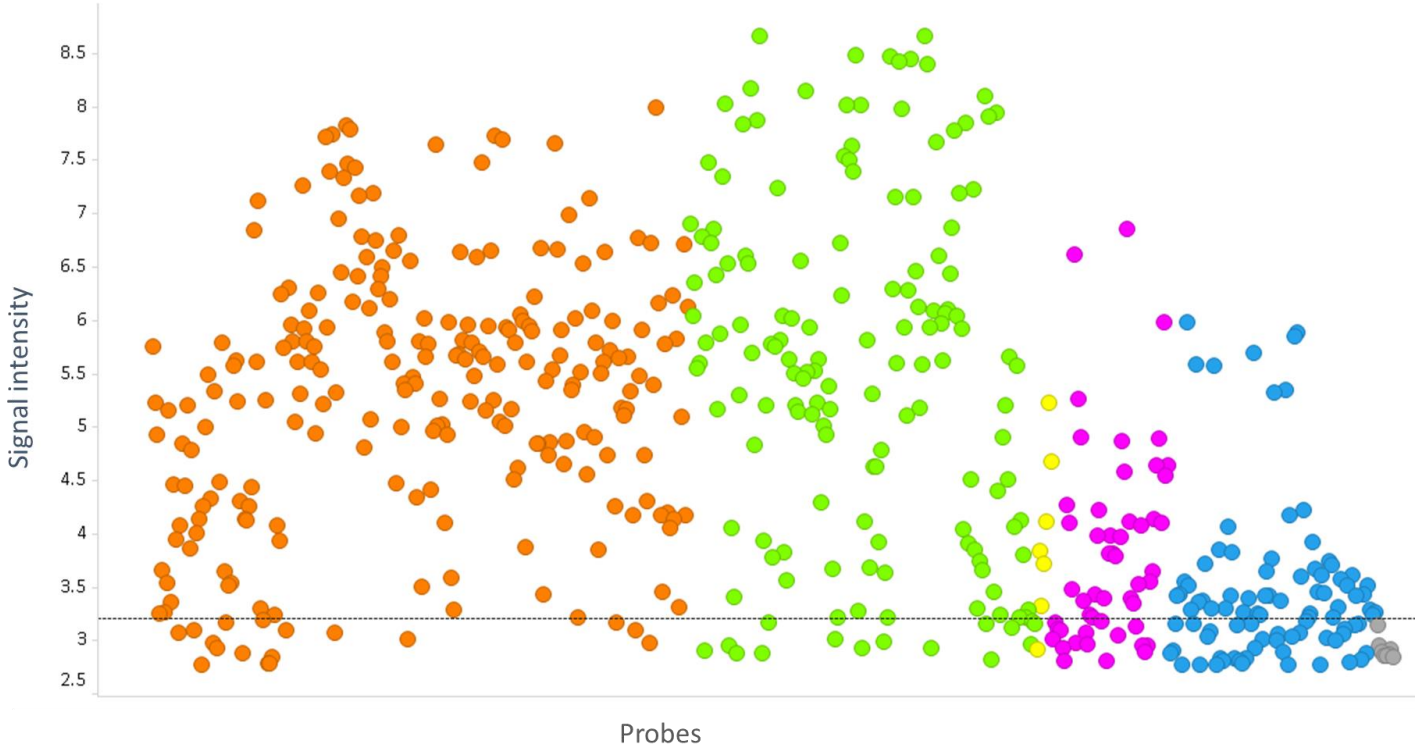


Figure 6.15. Normalised signal intensities following hybridisation of labelled DNA from five pathogens to Agilent platform (multiplex PCR- 2nd set).

Orange spot: *B. pilosicoli/ hyodysenteriae*, Green spot: PEDV, Yellow spot: P. rotavirus, Purple spot: *E. acervulina*, Blue spot: *C. jejuni/ coli,* Grey spot: Cross-hybridising probes from other pathogens – Scatter plot was generated with TIBCO® Spotfire®. Slightly less than 50% of the total number of probes were specific to *Brachyspira* species (217), 136 probes were positive for PEDV, 6 for Rotavirus, 35 for *Eimeria* and 57 for *C. jejuni/coli.* The cut-off (3.21) is represented by a horizontal dashed line.

Among all positive probes (n=488) (Table 6.5), 46.65% (n=209) of probes designed with eArray were able to hybridise to targets, a proportion of 27.90% (n=125) was achieved by UPS probes and 18.75% (n=84) by GoArray probes. Only 6.70% (n=30) of probes designed with Picky were able to identify the targets correctly.

Table 6.5. Number of detected pathogens per software in multiplex_2

Software	*B. pilo/ B. hyo*	PEDV	P. rotavirus A	*Eimeria acervulina*	*C.jej/ C. coli*	Total
eArray	122	38	4	14	31	209
UPS	42	59	2	10	12	125
Picky	5	16	0	5	4	30
GoArray	48	23	0	6	7	84
Total	217	136	6	35	54	**488**

6. 3. 1. 3. Probe performance by software.

Probe signal intensities were again compared according to their respective software design. Table 6.6 shows the number of positive probes for each reference strain tested per software, which were not significantly different. However, it seems that eArray and UPS generated the highest number of hybridised probes to their targets, with eArray probes being the best performing probes followed by UPS, Picky and GoArray.

Table 6.6. Number of positive probes per pathogen and software

	UPS	Picky	eArray	Goarray
P. rotavirus	3	0	4	0
TGEV	15	12	6	0
PEDV	83	17	51	27
PCV	58	22	41	8
C. difficile/perfringens	4	0	13	5
S. Typhimurium/Enteritidis	4	3	26	2
C. jejuni/coli	31	12	16	12
E. coli F4 /F5	8	7	5	1
B. pilosicoli/ hyodysenteriae	5	2	96	44
Eimeria acervulina	16	4	18	7
Bovine cytochrome b	4	0	27	0
Porcine cytochrome b	2	0	5	4
Multiplex 1 pathogens	73	21	80	38
Multiplex 2 pathogens	125	30	209	84
Total	**421**	**130**	**597**	**232**

As indicated earlier, false positive probes were also compared by software design In Table 6.7, are represented the number of false positive probes, which are the result of cross-hybridisation.

Probes that showed the highest total number of cross-hybridisation (Table 6.7) were the ones designed with UPS and GoArray, followed by Picky, then eArray. Misidentifications between the software were not statistically different.

The overall percentage of correct positive probes identified by UPS, Picky, eArray and GoArray were 30.74%, 32.38%, 39.04% and 34.38% respectively. Falsely positive probes totaled respectively 0.40%, 0.82% and 0.94% for UPS, Picky and GoArray. No false positives were registered among eArray-designed probes.

Table 6.7. Number of falsely positive probes per pathogen and software

	UPS	Picky	eArray	GoArray
P. rotavirus	0	0	0	0
TGEV	0	0	0	0
PEDV	0	0	0	0
PCV	1	1	0	0
C. difficile/perfringens	0	0	0	0
S. Typhimurium/Enteritidis	0	1	0	2
C. jejuni/coli	0	0	0	0
E.coli F4/F5	0	0	0	0
B. pilosicoli/ hyodysenteriae	0	0	0	0
Eimeria acervulina	1	0	0	0
Bovine cytochrome b	1	0	0	1
Porcine cytochrome b	0	0	0	0
Multiplex 1 pathogens	0	1	0	0
Multiplex 2 pathogens	0	0	0	0
Total	**3**	**3**	**0**	**3**

6. 3. 2. Sensitivity of Agilent platform

The sensitivity testing of the array was assessed using two enteric pathogens; *E. coli* F5 (K99) and PCV2. Hybridisations were performed using labelled PCR products of the F5 gene. The DNA was initially extracted from spiked faeces with 10-fold dilutions 10^{-2} (4.17×10^{10} copy number) to 10^{-5} (4.17×10^{7} copy number) of a culture of *E. coli* F5. For PCV, donated nucleic acid was two-fold diluted, and amplified with specific primers, labelled and hybridised to the array. Porcine host DNA was spiked with PCV2 samples and bovine host DNA

with *E. coli* K99. The aim of the spiking was to mimic a natural infection, eventually to observe whether any cross-reactivity with the host DNA could occur during hybridisation. The results of PCV2 hybridisation was illustrated in Table 6.8 which clearly shows that no detection of the virus was observed beyond a template concentration of 2 ng that corresponds to copy number 2.065 x 10^9, with a total number of 35 positive PCV probes .

Table 6.8. PCV sensitivity test of Agilent platform

	2 ng	1 ng	0.5 ng	0.25 ng
No hybridised PCV probes with signal intensities above the cut-off	35	0	0	0

Figure 6.16 shows the hybridisation profile with copy number 2.065 x 10^9 (A) and 1.03 x 10^9 (B) of PVC2. With the sample spiked with host porcine DNA, only one cytochrome b-specific probe among the highest signal intensity values showed an intensity of 3.14 . At lower pathogen DNA concentrations (1 ng (1.032 x 10^9), 0.5 ng (5.164 x 10^8 and 0. 25ng (2.582 x 10^8) (Fig. 6.16 -B and 6.17), only host DNA was detected with the highest signal intensities being recorded with pathogen DNA concentration at 0.5 ng and 0.25 ng (5.164 x 10^8 and 2.582 x 10^8 copies respectively).

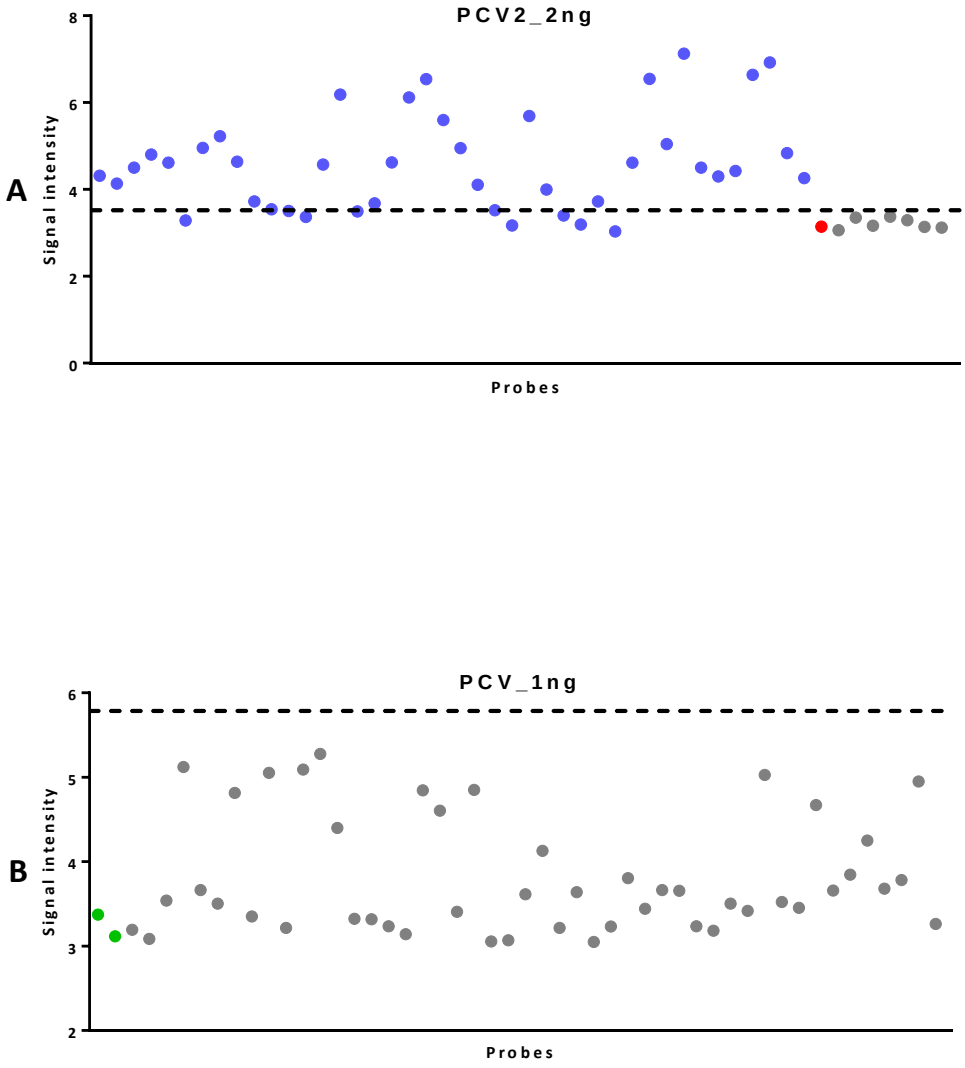


Figure 6.16. Normalised signal intensities profile following hybridisation with 2 ng (A) and 1 ng (B) of PCV2 to Agilent platform

Blue spot: PCV, Green spot: Porcine cytochrome b, Grey spot: Cross-hybridising probes from other pathogens. The probes showed high signal intensities when 2.065 x 10^9 copy number (2ng of PCV) were hybridised to the array (A). (B) shows only two specific probes to the host DNA (Green spots). Signal cut-off of 3.58 for PCV_2ng and 5.83 for PCV_1ng is represented by a dashed horizontal line.

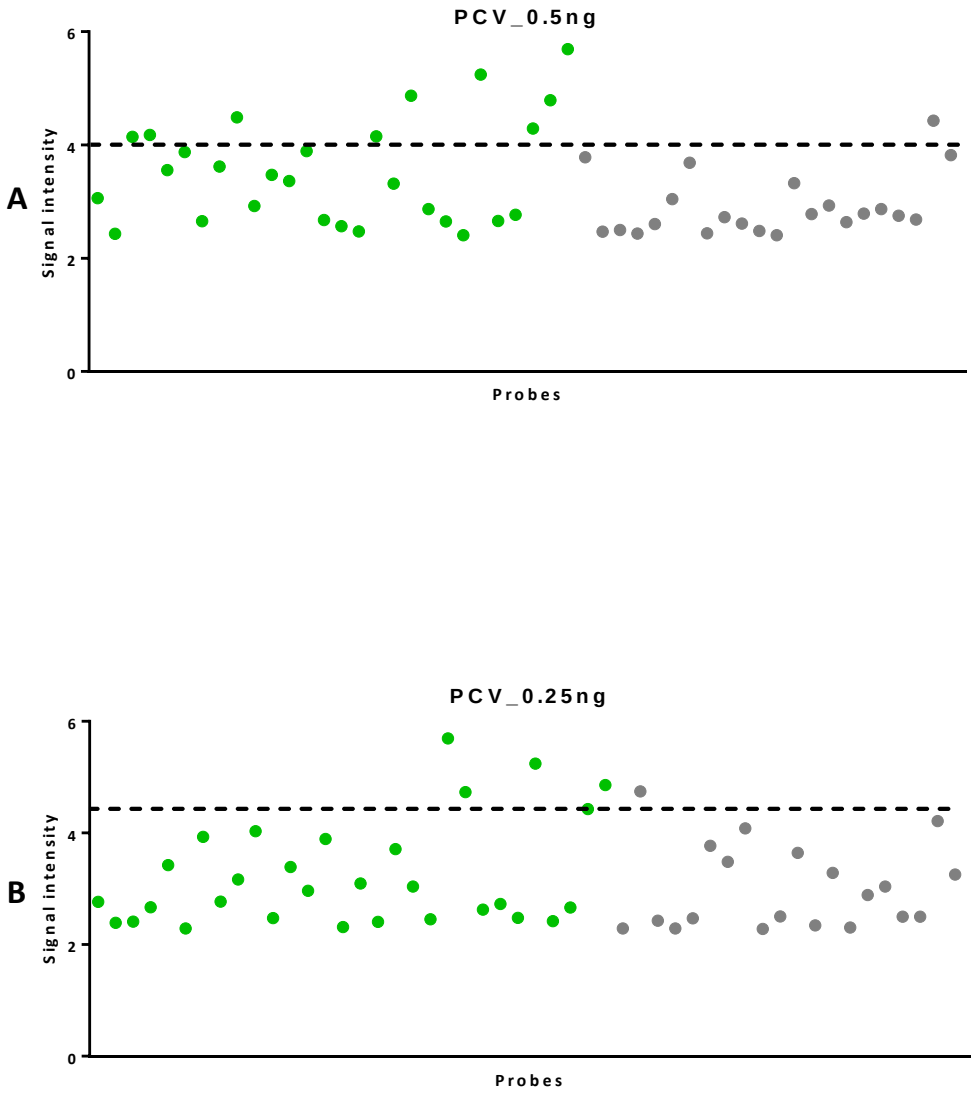


Figure 6.17. Normalised signal intensities profile following hybridisation with 0.5 ng (A) and 0.25 ng (B) of PCV2 to Agilent platform

Green spot: Porcine cytochrome b, Grey spot: Cross-hybridising probes from other pathogens. No PCV was detected with a DNA concentrations of 0.5 ng (5.164×10^8 and 0. 25ng (2.582×10^8). However 9 and 5 host cytochrome probes had signals above the cut-off for PCV-0.5ng and PCV-0.25ng respectively. The signal cut-off of 4.08 for PCV_0.5ng and 4.43 for PCV_0.25ng is represented by a dashed horizontal line.

To test the sensitivity of the array compared to PCR, qPCR and bacterial culture, spiked bovine faecal samples with *E. coli* F5 (K99) serial dilutions were used for

the detection of the fimbrial subunit gene by PCR, qPCR and array hybridisation; the bacterial count per ml was also determined. A negative control and non-spiked faeces were also introduced in all assays with the exception of the array hybridisation assay.

The PCR gel image (Fig. 6.18) shows three bands that correspond to the expected product size of 1120 bp. Agarose gel electrophoresis was able to detect the amplified fimbrial subunit gene down to a copy number of 4.17 x 10^9. No visible bands were noticed after amplification of samples spiked with K99 dilutions -4, -5 and -6. Equally no amplification was observed in the negative (non-template) control sample and non-spiked faeces, indicating that initially the faeces were free of *E. coli* F5.

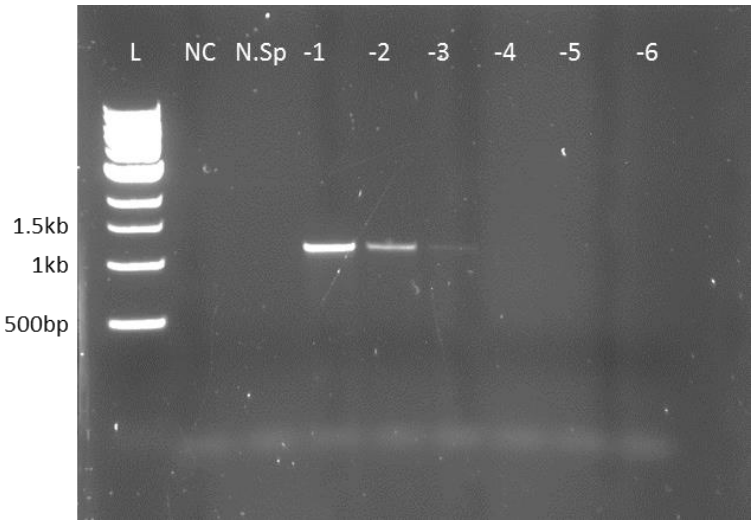


Figure 6.18. Agarose gel electrophoresis image of amplified fimbrial subunit gene in serial dilutions of *E. coli* F5 in spiked bovine faeces

L: 1kb DNA ladder, NC: negative control, N.Sp: non-spiked, -1 to -6: serial dilution of *E. coli* F5. PCR product size: 1120 bp. Copy number: -1: 4.17 x 10^{11}, -2: 4.17 x 10^{10}, -3: 4.17 x 10^9, -4: 2.08 x 10^8, -5: 4.17 x 10^7, -6: 4.17 x 10^6. Two clear bands and a faint band appeared for dilutions -1 and -2 and -3 respectively.

The real-time PCR results showed a specific amplification of the targeted gene

F5 in spiked faeces with dilutions -1 to -6 (Fig. 6.19). The gene was detectable at the 27th cycle for dilution -1, 30th, 33rd and 37th respectively for dilutions -2, -3 and -4. No amplification of the F5 gene was noticed for the negative control, non-spiked faeces and dilutions -5 and -6. The detection limit was 2.08 x 10^8 copies.

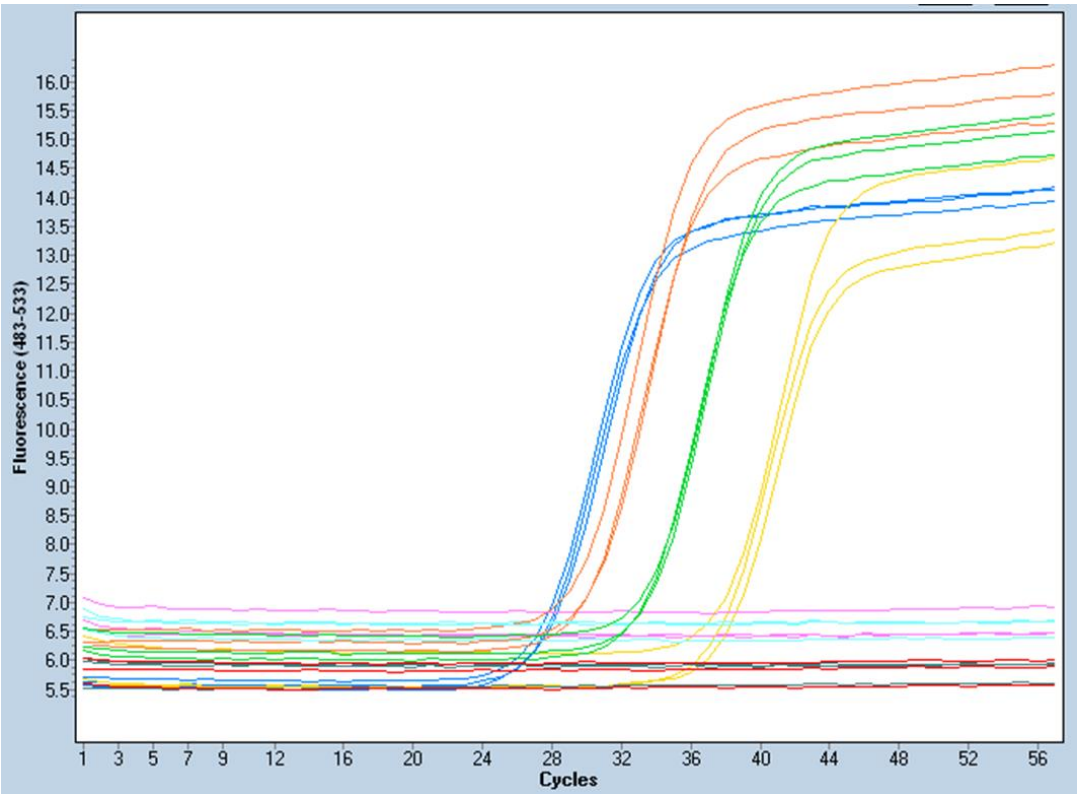


Figure 6.19. Real-time PCR of amplified F5 gene in spiked bovine faecal samples

Blue curve: dilution -1 (4.17 x 10^{11} copies) , Orange curve: dilution -2 (4.17 x 10^{10} copies), Green curve: dilution -3 (4.17 x 10^9 copies), Yellow curve: dilution -4(2.08 x 10^8 copies), Dark green curve: dilution – 5 (4.17 x 10^7 copies), Red curve: dilution -6 (4.17 x 10^6 copies).

Array hybridisation of spiked faeces with dilutions -2 to -5 are illustrated in Fig. 6.20 and 6.21. A total number of 70 spotted probes were specific to the *E. coli* fimbrial gene F5 on the array and were thus expected to hybridise. The overall

signal intensities were relatively low compared to reference strain signals. In the sample spiked with *E. coli* F5 dilution -2, only 12 probes showed signals that varied from 3.81 to 6.06. For the spiked sample with dilution -3, 11 probes were considered as positive. However for dilution -4 and -5, no detectable hybridisation above the cut-off was observed when the faecal sample was spiked with *E. coli* F5. Most of the probes that cross-hybridised with targets were the 16S rRNA probe. For instance 30 probes out of 37 were 16S specific probes that cross hybridised with *E. coli* F5 gene in dilution -4.

Four approaches were used to control the array-based detection of *E. coli* F5: plate bacterial count, PCR, real-time PCR and array hybridisation. Viable counting achieved the highest sensitivity, detecting 61 CFU at dilution -5, followed by the real-time PCR that amplified the target gene with less than 2.08 x 10^8 copies, equivalent to dilution -4. The PCR amplicon was detected by electrophoresis down to 4.17 x 10^9 copies, equivalent to dilution -3. The microarray seemed to have comparable sensitivity to real-time PCR (dilution -4 – 2.08 x 10^8) of the target gene present in the sample detected. It is important to note that the copy number was based on the amount of DNA in the sample prior to amplification.

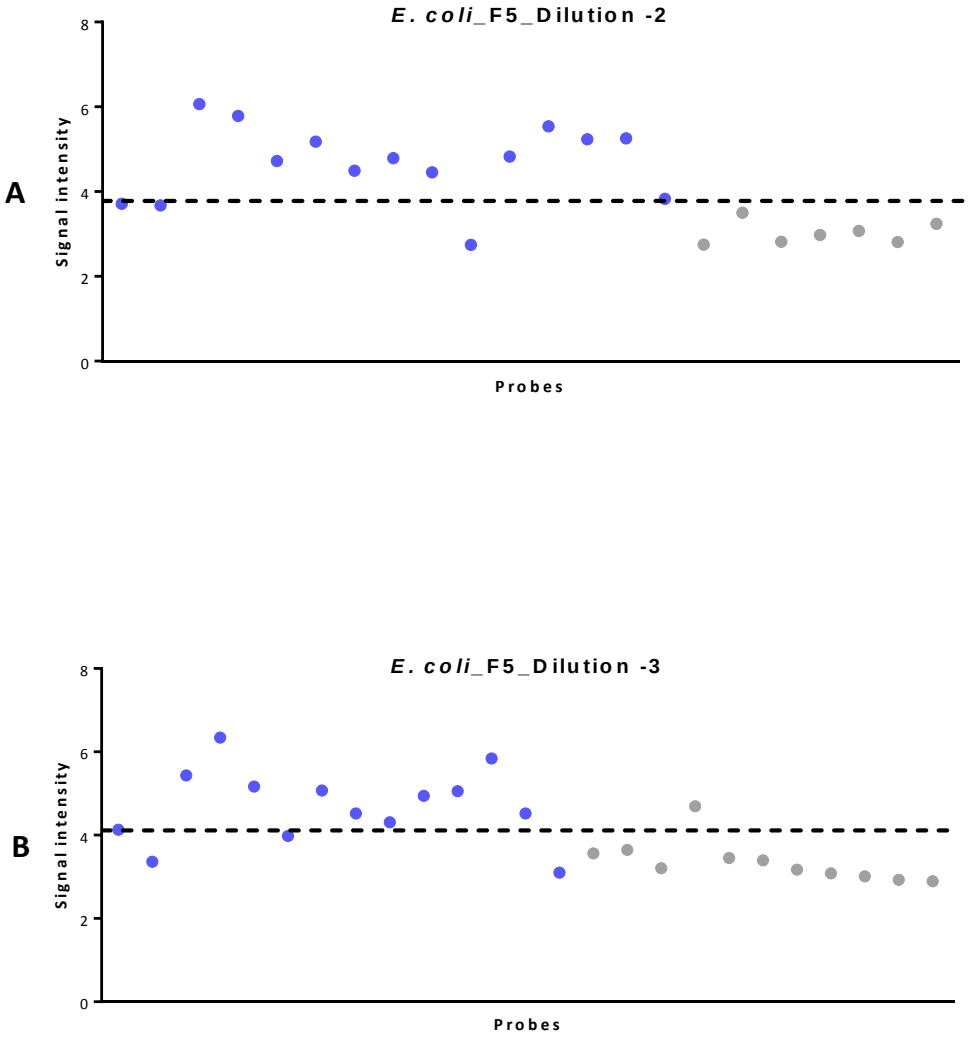


Figure 6.20. Hybridisation profile on Agilent platform with amplified fimbrial subunit gene in bovine faeces spiked with *E. coli* F5 at dilution -2 (copy number 4.17 x 10^{10}) (A), and -3 (copy number 4.17 x 10^{9}) (B)

Blue spot: F5 specific probe, Grey spot: cross-hybridising probes from other pathogens. Respectively 14 and 11 probes had signal intensities above the cut-off (dashed horizontal line) of 3.81 (dilution -2 - copy number 4.17 x 10^{10}) and -3 (copy number 4.17 x 10^{9}).

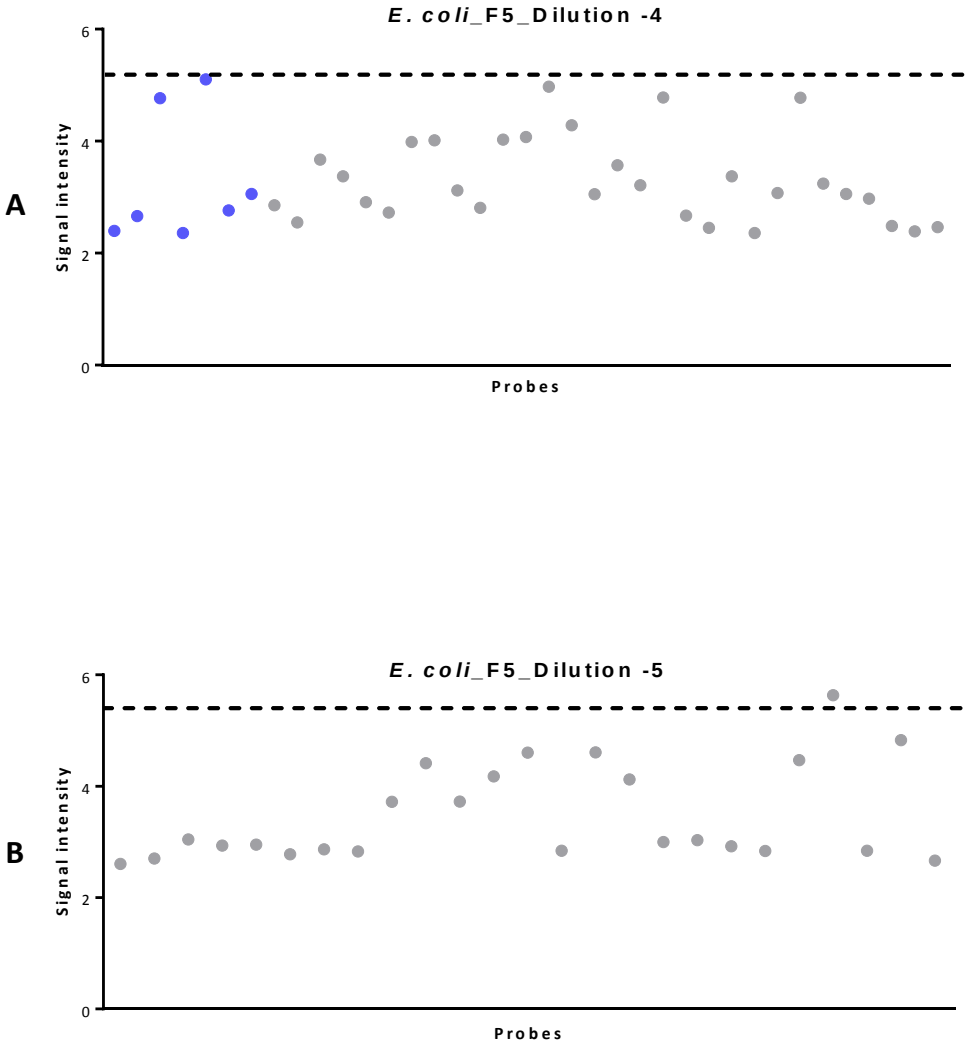


Figure 6.21. Hybridisation profile on Agilent platform with amplified fimbrial subunit gene in bovine faeces spiked with *E. coli* F5 at dilution -4 (copy number 2.08 x 10^8) (A), and -5 (copy number 4.17 x 10^7 copies) (B)

Blue spot: F5 specific probe, Grey spot: cross-hybridising probes from other pathogens. None of the PCV probes were positive although two probes showed signals of 5.10 and 4.76 (blue spots (A)), still below the cut-off. Dashed horizontal line represents a signal cut-off of 5.23 and 5.42 for dilution -4 (2.08 x 10^8 copies) and -5 dilution (4.17 x 10^7 copies), respectively.

6. 3. 3. Hybridisation with clinical samples

6. 3. 3. 1. Bovine samples

Four faecal samples of diarrhoeic calves were subjected to array hybridisation using the Agilent platform following hybridisation. All probes deriving from 16S rRNA , 18S rRNA, 23S rRNA and ITS regions were removed from the data output. The 16S rRNA gene is not precisely indicative of the presence of a specific bacterial species in the sample. In fact, it might be the 16S rRNA of any bacteria as the whole gene has been amplified, and not the most variable discriminatory region of the gene coding for the small subunit of the ribosomes. Additionally, all cross-hybridising probes with tested reference strains were eliminated from the analysis.

These clinical samples were considered positive to a pathogen, when a set of probes representing the same pathogens showed the highest signal intensities (above the cut-off limit), with many probes specific to several genes of the same pathogen and in particular to the amplified sequence.

Hybridisation assay of samples 1/3 (Fig. 6.22) showed mainly the presence of two species of bacteria hybridised to three probes and four virus probes, although six probes had their signals below the cut-off. This sample, with only one unique probe showing above cut-off signal, was considered as negative to the targeted pathogens. The *Yersinia*-probe with a signal of 1.76 was specific to type 3 secretion system needle.

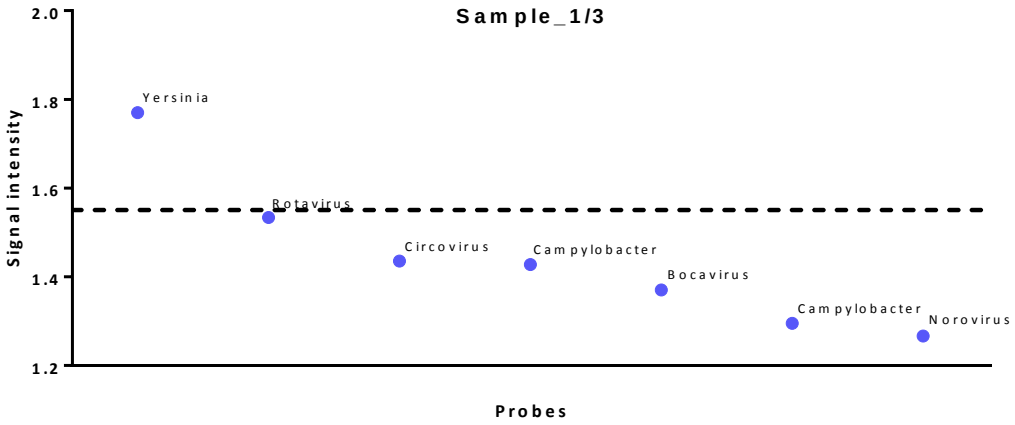


Figure 6.22. Normalised signal intensities of bovine faecal sample 1/3 on Agilent platform

Probes represented by blue spots were designed from virulence genes for bacteria and structural and non structural coding genes for viruses. It clearly appears that this sample was negative to major enteropathogens. Very few representative probes were present. Dashed horizontal line represents a signal cut-off of 1.55.

Sample 1 hybridisation results (Fig. 6.23), revealed more consistent comparison between the array and the PCR. Indeed, ETEC F5 probes were positive after hybridisation symbolised by blue dots in the figure, the highest signal intensity being 8.83. Among the 16 ETEC F5 specific probes, 14 had their signals above the threshold of 3.25.

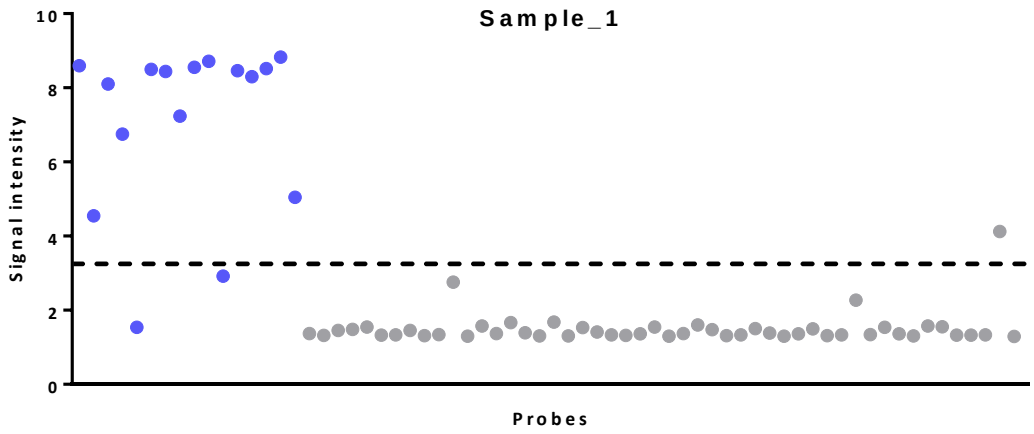


Figure 6.23. Normalised signal intensities of bovine faecal sample 1 on Agilent platform

Blue spot: ETEC F5, Grey spot represent viruses including BVDV, coronaviruses, rotaviruses and noroviruses, they also include antibiotic resistance probes. High signal intensities of ETEC F5 probes (blue spot) denote the presence of the pathogen in this sample. A neat distinction of signal intensities of the first 14 probes (blue spots) is observed, ranging from 6.75 to 8.83. Dashed horizontal line represents a signal cut-off of 3.25.

A confirmatory PCR was carried out for the detection of ETEC F5 in this sample. Specific primers were used to obtain amplicons with a size of 1120 bp. Figure 6.24 shows the presence of the fimbrial unit F5 in sample 1 with the expected PCR product size.

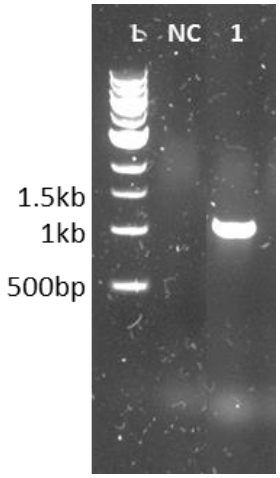


Figure 6.24. Agarose gel electrophoresis image of confirmatory PCR of sample 1

L: 1kb DNA ladder, NC: Negative control, 1: ETEC F5. Sample 1 shows the presence of ETEC F5 with an expected size of 1120 bp.

Two other samples (20T and 55) were hybridised to the array. Sample 20T showed a more diverse presence of bacterial, viral and parasitic species (Fig. 6.25). Viral species included members of rotavirus, BVDV, parvovirus, circovirus and coronavirus. Bacterial species comprised *Clostridium difficile* which was identified based on toxin A *tcdA* only. For this particular bacterium, among 40 *C. difficile*-specific probes, 26 corresponded to *tcdA*, 7 to *Clostridium* antibiotic resistance genes (Chloramphenicol, erythromycin and tetracycline). The remaining 7 probes were specific to toxin B *tcdB* and binary toxin *cdt.* Rotavirus was also a pathogen that has a significant number of probes (26) with a signal intensity varying from 2.36 to 8.29.

Other microorganisms were also detected in this sample, including Gram negative aerobic and anaerobic bacteria, with a majority of facultative anaerobic bacteria and *E. coli.* The probes were in this case hybridising to virulence and antibiotic resistance genes that could be carried by any commensal intestinal bacteria. Bovine cytochrome b was also identified with eight positive probes.

Unfortunately none of these organisms was confirmed by PCR.

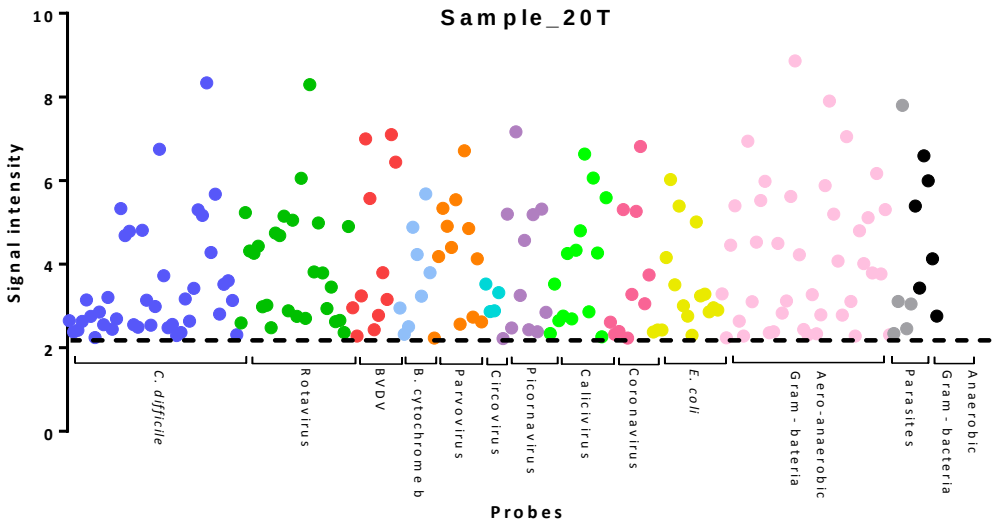


Figure 6.25. Normalised signal intensities of bovine faecal sample 20T on Agilent platform

At least ten different microbial species were present in this sample, however with varying representative probes, *C. difficile* and Rotavirus were the most represented pathogens. Other viruses such as BVDV, Parvoviruses, Circoviruses, Picornaviruses, Caliciviruses and coronaviruses were identified, nevertheless by few probes. The high background noise contributed largely in the low cut-off value represented by a dashed horizontal line at a signal intensity of 2.22.

In sample 55 (Fig. 6.26), hybridisation results showed a relatively large number of ETEC F5 probes (16) with signal intensities of 4.76 – 2.06, with 14 probes above signal cut-off 3.45. Other hybridised positive probes were represented by viral species (rotavirus, coronavirus, circovirus, picornavirus and norovirus). Interestingly, among pathogen specific probes, 11 probes corresponded to *E. coli* attaching effacing gene (eae).

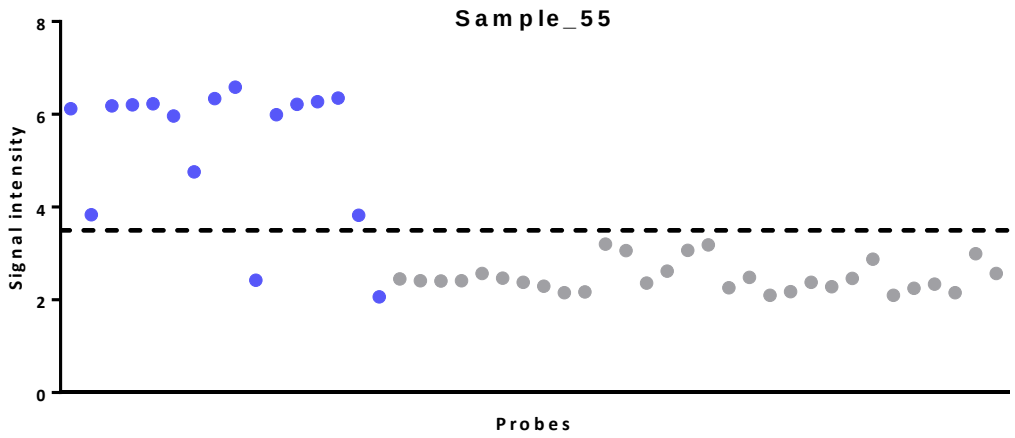


Figure 6.26. Normalised signal intensities of bovine faecal sample 55 on Agilent platform

Blue spot: ETEC F5. Grey spots represent *E. coli eae* genes, but also few probes from viruses such as rotavirus, coronavirus, circovirus, picornavirus and norovirus, however their corresponding signal intensities remain below the cut-off of 3.45, illustrated by a dashed horizontal line.

The same approach was adopted for sample 55, for which a confirmatory PCR was carried out (Fig. 6.27). The gel image clearly demonstrated the presence of the fimbrial unit F5 gene (1120 bp) in the sample which is in accordance with the microarray results.

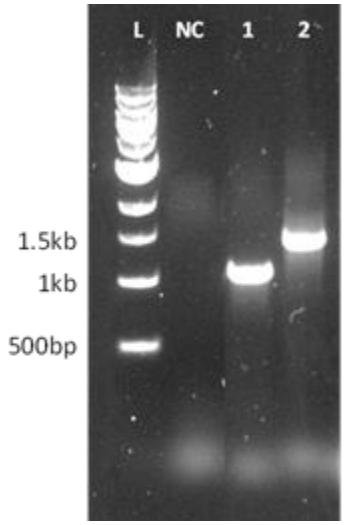


Figure 6.27. Agarose gel electrophoresis image of confirmatory PCR of sample 55

L: 1kb DNA ladder, NC: Negative control, 1: ETEC F5, 2: 16S rRNA. The figure shows presence of ETEC F5 in sample 55 with an expected size of 1120 bp. The band in lane 2 corresponds to bacterial 16S ribosomal RNA amplicon of 1494 bp.

6. 3. 3. 2. Porcine samples

A total of four porcine faecal samples were hybridised on the Agilent platform. Piglets were diarrhoeic and a high mortality rate was recorded. The samples were initially searched for major pathogens in a separate study using ELISA and targeting Porcine rotavirus A and *C. difficile*. In addition to pathogens already shown to be present by the provider using an ELISA, Porcine kobuvirus has been serendipitously identified by PCR in some samples. The samples were subjected to a multiplex PCR targeting Porcine rotavirus A, Porcine kobuvirus, *C. difficile* 16S rRNA, *C. difficile* toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdt*). The multiplex PCR products were labelled and hybridised to the array. As multiple pathogens /genes were targeted in porcine samples and unlike the bovine clinical samples, porcine samples contained specific pathogens that were confirmed by PCR (Fig. 6.28) and by ELISA, consequently a higher number of probes, above the cut-off have been considered in the identification of the pathogens. Also all those considered positive and negative, were illustrated in the figures to evaluate the extent of cross-hybridised probes, the latter could also be assumed to belong to one of the PCR-targeted pathogens, particularly bacterial, as many genes were more likely to have shared regions amongst the bacteria and parasites. These can be transferred by transduction, transformation and conjugation.

As mentioned before, a multiplex PCR reaction was performed with four porcine samples (samples 8, C, 4B and 7B) and examined by electrophoresis on a 1.2% agarose gel (Fig 6.28).

The figure confirms the presence of *C. difficile* in samples 8 and particularly C and 4B with an expected PCR product size of 1469 bp for the 16S rRNA, but also the presence of a *tcdA* with a 1437bp expected amplicon size. These two PCR products have nearly the same size, which means that although two clear distinct bands were not visualised on the gel, they might have been amplified but were overlapping, thus producing a unique band.

Sample 8 contained *C. difficile* and Porcine rotavirus A with the expected size of amplicon, 1469 bp, 1437 bp and 521 bp respectively for *C. difficile* 16S rRNA, *tcdA* and P. rotavirus.

Samples C and 4B appeared to contain the same pathogens; indeed the gel electrophoresis image shows that both samples contained *C. difficile*. Two unexpected bands (indicated with yellow arrows) were present and correspond to 550-600 bp and 900-1000 bp.

Finally, sample 7B shows a single visible band (indicated with a white arrow) corresponding to the expected PCR product size of Porcine kobuvirus (803 bp).

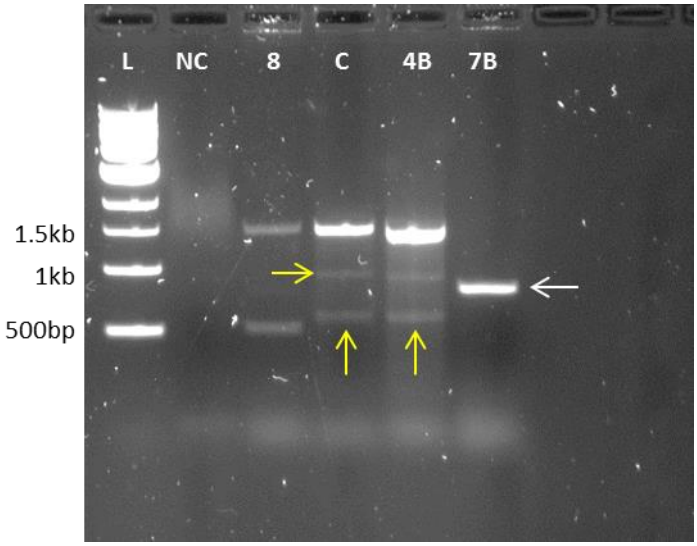


Figure 6.28. Agarose gel electrophoresis image of confirmatory multiplex PCR for porcine clinical samples

L: 1 kb DNA ladder, 8, C, 4B and 7B: samples. White arrow indicates a faint band corresponding to P. kobuvirus product size, Yellow arrows indicate unexpected bands. Samples 8, C and 4B show bands corresponding to *C. difficile* 16S rRNA (1469bp). Samples 8 and 7B contain respectively P. rotavirus (521bp) and P. kobuvirus (803bp). *C. difficile* 16S rRNA: 1469 bp, *C. difficile tcdA*: 1437 bp, *C. difficile tcdB*: 1266 bp, *C. difficile* binary toxin *cdt*: 1774 bp, P. kobuvirus VP1: 803 bp, P. rotavirus VP7: 521 bp.

For the four porcine samples, the threshold values were calculated as mentioned earlier. As indicated for bovine samples, signal intensity data from specific probes of 16S, 23S and ITS probes have been removed as they would affect the cut-offs considerably. This was carried out despite the hybridised multiplex PCR products on the array comprised the 16S rRNA.

The results of sample 8 hybridisation on the array (Fig. 6.29) suggested a high dominance of *C. difficile* followed with 34 probes, Porcine rotavirus A with 29 probes and Porcine kobuvirus with 17 probes above the cut-off signal of 3. Again, it is important to point out that all *C. difficile* probes in the figures are representative of toxin A and binary toxin genes.

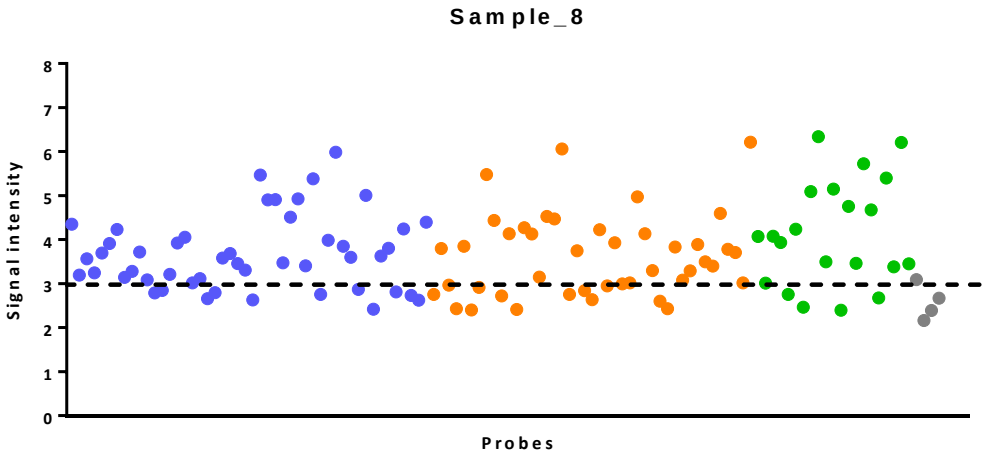


Figure 6.29. Normalised signal intensity of hybridised sample 8 onto Agilent platform

Blue spot: *C. difficile tcdA* and *cdt*, Orange spot: P. rotavirus A, Green spot: P. kobuvirus, Grey spot: Gram negative bacteria. A total of 46 toxin A (tcdA) and binary toxin (cdt) probes had a signal of 2.41 to 5.98. However 34 were above the threshold of 3.00. Rotavirus probes counted 43 probes with 29 above cut-off and kobuvirus 21 with 17 over cut-off. Dashed horizontal line represents the signal cut-off.

Array hybridisation of sample C displayed a total of 55 probes specific to *Clostridium* toxin A and binary toxin (Fig. 6.30) with signal intensity fluctuating between 2.23 and 5.26. Above cut-off (3.72), 26 and 5 probes were specific to *tcdA* and *cdt* respectively. Other viral and bacterial species were also present but their signal intensity remainded lower than the cut-off, except from one P rotavirus probe. Four probes were specific to rotavirus with intensities varying from 2.25 to 4.67.

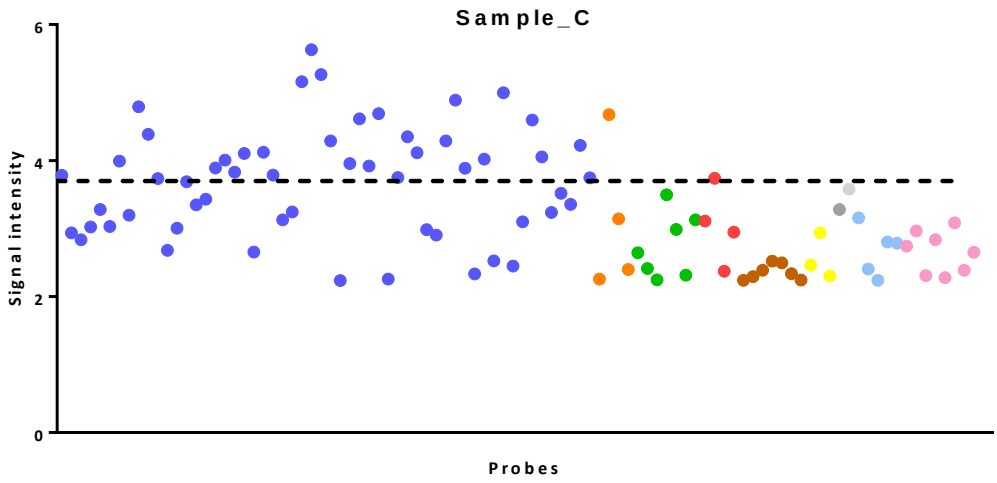


Figure 6.30.Normalised signal intensity of hybridised sample C onto Agilent platform

Blue spots: *C. difficile tcdA* and *cdt*, Orange spot: Rotavirus, Green spot: Coronavirus, Red spot: Norovirus, Brown spot: Picornavirus, Yellow spot: Bocavirus, Grey spot: Circovirus, Light grey: *Cryptosporidium*, Light blue spot: Bacterial virulence markers, Pink spot: Antibiotic resistance markers. More than 30 *tcdA* and *cdt* probes had their intensities above the cut-off of 3.72, illustrated by a horizontal dashed line.

Sample 4B array hybridisation (Fig. 6.31) also showed a substantial presence of *C. difficile* with 49 positive probes composed exclusively of probes designed from *C. difficile* toxin A and binary toxin. Interestingly, this sample exhibited the presence of *B. fragilis*, and specifically the presence of the clindamycin resistance gene (*ermF*) with 17 probes corresponding to the gene. In this sample, the high background noise contributed noticeably to increase the cut-off threshold which reduced the number of probes above cut-off signal intensity to only eight, five of which were specific to toxin A and three to the binary toxin. The remaining probes were specific to porcine cytochrome b, bocaviruses, noroviruses and antibiotic resistance genes of *E. coli*. Eight probes reacted with the host cytochrome b gene, this gene was not subjected to specific PCR. Nevertheless it might have been amplified during the random amplification step.

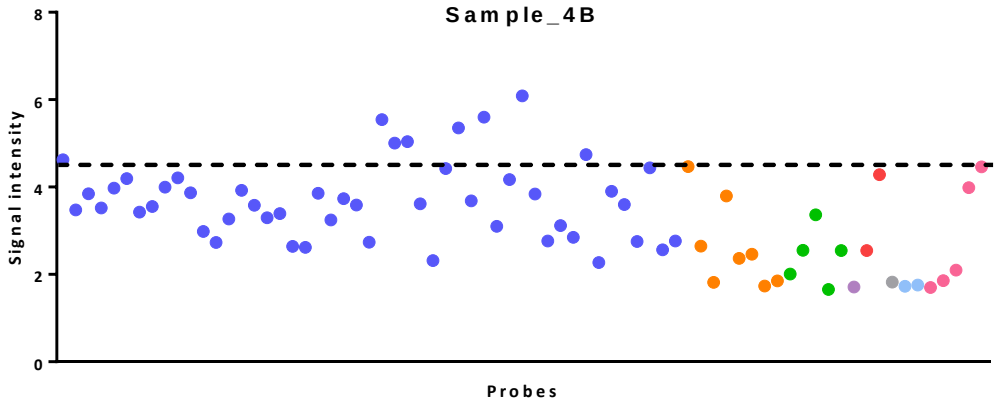


Figure 6.31. Normalised signal intensity of hybridised sample 4B onto Agilent platform

Blue spot: *C. difficile tcdA* and *cdt*, Orange spot: Host cytochrome b, Green spot: Bocavirus, Purple spot: Rotavirus, Red spot: Norovirus, Grey spot: Circovirus, Light blue spot: Bacterial virulence markers, Pink spot: Antibiotic resistance markers. Eight *tcdA* and *cdt* probes had a signal over 4.57 (represented by horizontal dashed line), however a total of 49 probes had an intensity between 2.26 and 6.08.

Array interrogation with sample 7B (Fig. 6.32) revealed 38 Porcine kobuvirus probes that hybridised to the virus genome. Probes with signals above the cut-off of 7.49 counted 21. Also one probe yielded a signal of 5.50 for Porcine rotavirus A and five to *C. difficile* tetracycline resistance with relatively high signals (4.93 – 6.51). As the previous sample, a high background noise was observed for this sample which affected the cut-of value. Despite being below the cut off, many probes showed signals from 4.91 to 7.25, with in total of six species of viruses (Norovirus, coronavirus, parvovirus, bocavirus, circovirus, and pestivirus). Seven probes were specific to bacterial virulence genes and 13 to antibiotic resistance genes.

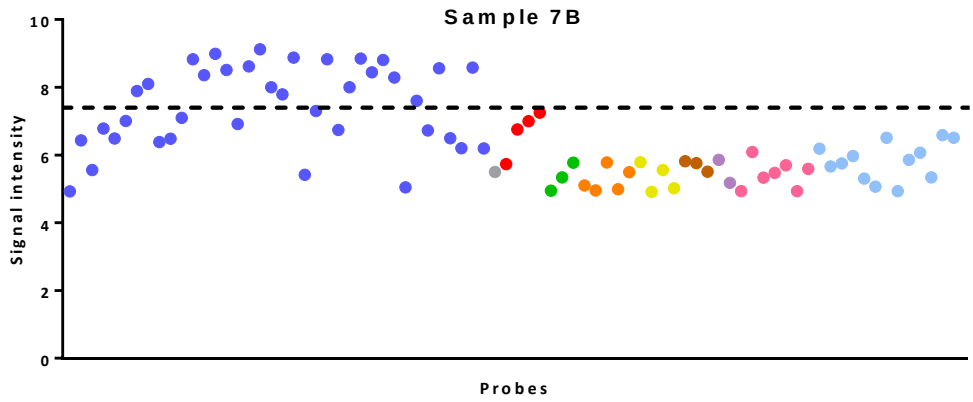


Figure 6.32. Normalised signal intensity of hybridised sample 7B into Agilent platform

Blue spot: P. kobuvirus, Grey spot: Rotavirus, Red spot: Norovirus, Green spot: Coronavirus, Orange spot: Parvovirus, Yellow spot: Bocavirus, Brown spot: Circovirus, Purple spot: Pestivirus, Pink spot: Bacterial virulence markers, Light blue spot: Antibiotic resistance markers. Above the signal intensity cut-off, a total of 21 probes specific to P. kobuvirus had a signal between 7.59 and 9.11. Dashed horizontal line represents the signal cut-off of 7.49.

6. 4. Discussion

The development of an array-based approach to diagnosis and surveillance requires a long term strategy involving the parallel development of a more targeted smaller array suitable for high-throughput screening together with a large pan-pathogen array containing a much wider selection of probes which might be used for confirmation studies in outbreaks but also for a more refined in depth analysis of individual samples and which may contain new genetic combinations, including new pathogens.

The Agilent microarray platform supports high density oligonucleotides, generally of 60-mer size. The format 4x44k has been used in this study with 15993 probes spotted in duplicates on a glass slide.

The validation of enteric probes using the Agilent platform in this study followed the same approach as employed for viral enteric probes on the Alere platform.

The specificity of the platform was assessed using four viruses, seven bacteria and one parasite. Two multiplexed target genes for five different pathogens each were also used to test the detection specificity of this platform. The results showed that TGEV, PEDV and PCV were identified appropriately by Agilent microarray. This was not the case for Porcine rotavirus A, for which only seven probes were able to hybridise, however their signal intensities were the highest (6.8-8.0). Porcine rotavirus A amplification produced a fragment of VP7 gene, which has been showed to encompass 27 G genotypes (Matthijnssens et al., 2011). The probe design was based on all porcine genotypes sequences

deposited in GenBank at the time of the design, but rotavirus VP7 might belong to a non-covered genotype by the design and possibly the virus might have been subjected to reassortment of the genome segment, an event that has been reported by many studies (Trojnar et al., 2010; Lorenzetti et al., 2011; Papp et al., 2013). The almost identical performance of P. rotavirus probes with the Agilent array compared with the Alere array again suggests that closer attention to probe design including elimination of poor performing probes is an essential component of array improvement.

S. Typhimurium and *S.* Enteritidis showed a high level of cross-hybridisation, in favour of *S.* Enteritidis, as the probes were more specific to it than to *S.* Typhimurium. The main reason was that the hybridised target was nearly the entire 16S rRNA sequence, an approximatively 1500 bp sequence coding for the 16S ribosomal small subunit that has a high degree of conservation at the genus level, but also among all members of the *Enterobacteriaceae* family (Brenner, 1992). This downside was not observed in *C. difficile* and *C. jejuni* hybridisation results where they have been more accurately identified with a high number of species-specific probes for both species.

Host DNA is present in biological samples and represents a large amount of the extracted DNA (Feehery et al., 2013), consequently it is present and complicates downstream processes and reactions. Hybridisation of genomic host DNA through mitochondrial cytochrome b was aimed mainly to assess whether host DNA cross hybridises with non-host probes. The results showed positive hybridisation to host specific probes. Some bovine and porcine probes,

however, also cross-reacted with non-target probes, however their signals remained below the cut-offs. Indeed the cytochrome b gene possesses a highly conserved region of 359 bp and the identity of the whole sequence of bovine and porcine cytochrome b was 86.64% (Erwanto et al., 2012). As indicated in Chapter 5 it may be that host DNA containing a more balanced combination of genes would have provided a better spread of weaker signals against which pathogen signals could have been compared.

The *E. coli* fimbrial adhesins F4 and F5 array hybridisation led to simultaneous detection of virulence and antibiotic resistance genes. This indicates that genes that are harboured on the plasmid would, not surprisingly, also be amplified when the sample was prepared for the labelling step. Several authors have demonstrated that virulence factors as well as antibiotic resistance genes were carried on the same plasmid than adhesins F4 and F5 (Gonzalez and Blanco, 1985; Harnett and Gyles, 1985). Moreover, according to Partridge (2015), members of the *Enterobacteriaceae* family share considerable amounts of genetic material, that consist of genes captured from chromosomes of different species which are then transferred through plasmids. These plasmids have been demonstrated to mediate horizontal transfer between different species and genera of bacteria, depending on their host range (Thomas and Nielsen, 2005).

Based on the above, for *E. coli* F4 and F5, all probes corresponding to the *Enterobacteriaceae* family and other Gram negative/positive bacteria were considered as positive, as the virulence or antibiotic resistance gene might be

present on the plasmid. Eventually, if the gene is located elsewhere in the bacterial genome, it could also generate a positive hybridisation since a random amplification step was performed prior to labelling and can, to some extent, increase the chances for other genes to be identified.

B. pilosicoli has also been identified accurately by the array. Similarly, *E. acervulina* showed a high level of hybridised specific probes.

Amplicons from two multiplex reactions (multiplex_1 and multiplex_2) containing a mixture of five DNA templates of different pathogens each were hybridised onto the same array using Cy3 and Cy5 dyes. The results showed that the ten targets were correctly identified, albeit unevenly; the hybridisation clearly might be dependent on the amount of each DNA/cDNA in the sample and sensitivity studies are thus important as a validation step. Rudi et al. (2003) reported weak signal intensities when hybridised targets were diluted. It is also essential to point out that a hybridisation solution with multiple targets is a complex system where millions of targets are present and competition of targets for the binding to the probes is thus to be expected.

Moreover, the number of existing probes on the array is essential as a higher number of probes increases the chance of the target to be detected which then leads to a philosophical investigation of the approach to pathogen detection in terms of how many probes signify a positive detection. TGEV and *Eimeria* showed a low number of spots as they were the least represented among the 10 pathogens on the array with 151 and 107 probes, respectively. This was not applicable for all pathogens, since for instance the array carries 846 specific

probes to Porcine rotavirus A, B, and C. However, only seven probes were positive for Porcine rotavirus A. Interestingly, the same probes were positive when the pathogen was hybridised following singleplex amplification. This could be attributable to target single stranded DNA secondary structure that might have notably impacted the hybridisation efficiency (Lane et al., 2004).

It was clear that some probes for certain pathogens gave a uniformly lower signal intensity (e.g. TGEV). This could be explored in more detail in future in terms of probe selection.

Although multiplex PCR was performed for only five pathogens, a higher number with could be achievable with the necessary optimisations resulting in targeting other major enteropathogens that might be present in the sample. It Also offers the advantage of using smaller amounts of sample and to use fewer microarrays which reduces considerably the cost of the detection. Many studies have used multiplex PCR amplicons for microbial detection (Volokhov et al., 2002; Wilson et al., 2002; Jääskeläinen et al., 2006; Wang et al., 2011). These authors reported low signal intensities, false positive, cross-hybridisations and loss of sensitivity.

The use of specific amplification with multiplex PCR has been used in this chapter for experimental purposes. The work from the Alere array indicates advantages of using random over specific amplification, not least because we are unable to presume to know what pathogens are in any clinical sample.

Non-specific hybridisation could arise from non-specific PCR products generated during the multiplex reaction as no optimisation of the technique

was performed. Additionally, the random amplification step carried out prior to labelling might also increase the amount of non-target sequences in the sample. The 16S rRNA cross-reactivity between all bacteria is also a problem for this array design, which necessarily needs confirmatory post-hoc experiments. Eventually, discriminative detection of pathogen could also be based on amplified targets from other regions of the genome including specific virulence and antibiotic markers of the pathogens.

The sensitivity of the Agilent platform in discriminating descending amounts of viral (PCV-2) targets showed a low sensitivity of 2.065 x 10^9 copies as found with the Alere array (chapter 5). This can be attributed to a low efficiency of amplification in samples with less than 2 ng of viral RNA. Host DNA was spiked with viral nucleic acids to simulate a natural infection. It appears that at lower amounts of viral targets, porcine host DNA was more likely to hybridise to corresponding host probes, disadvantaging PCV2 hybridisation. This might be due to a competitive process for binding during hybridisation or may be the result of biased priming kinetics.

The detection limit of the microarray when spiked bovine faecal sample with *E. coli* F5 was hybridised seemed to be comparable to the qPCR technique detection limit of 2.08 x 10^8 copy number (dilution -4), followed by the PCR technique with 4.17 x 10^9 copy number (dilution -3). This was disappointingly poor and would have resulted in a repeated investigation given more time but certainly merits clarification in terms of technical faults which most likely is the basis of the poor sensitivity. The highest sensitivity was attained by the plate

count but this was also poorer than expected. The low sensitivity of the PCR might be the result of poor primer selection. Also, no optimisation was performed, as indicated earlier.

Further array evaluation was based on interrogation with four porcine and four bovine clinical samples. For what was a preliminary investigation the samples were subjected to multiplex PCR targeting major enteric pathogens in the bovine species and *C. difficile* and its toxins, Porcine rotavirus A and Porcine kobuvirus in the porcine samples.

All bovine sample hybridisation data analysis was based only on probes from virulence and antibiotic resistance genes and those designed from viral structural and non-structural gene sequences. Ribosomal specific probes, which were non-discriminatory for bacteria and parasites were removed from the data output.

Sample 1/3 showed the presence of only one probe corresponding to a type 3 secretion system, this is visibly not conclusive to assert the causality of the intestinal infection. On the contrary, samples 1 and 55 clearly showed the presence of *E. coli* F5 with the highest probe signal intensities which was also confirmed by PCR. Several studies demonstrated that the most common cause of neonatal diarrhoea in calves is *E. coli* that possesses adhesion antigen F5 (Younis et al., 2009, Bartels et al., 2010; Picco et al., 2015; El-Seedy et al., 2016). Sample 20T showed a multiple presence of species, with signal intensities higher than the cut-off. In terms of the number of representative probes of pathogens, it seems that *C. difficile* and Rotavirus counted the highest number

of probes, however, as no confirmatory PCR was carried out, it is consequently hypothethised that this sample might contain these two pathogens.

In porcine samples, six pathogens/toxins were targeted, Porcine rotavirus A (VP7), Porcine kobuvirus (VP1), *C. difficile* (16S rRNA), *C. difficile* toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdt*). The array successfully identified the pathogens, with samples 8 containing *C. difficile* and its toxins, A (*tcdA*) and binary toxin (*cdt*), Porcine rotavirus and Porcine kobuvirus. Both samples C and 4B contained *C. difficile*, although only eight *C. difficile* toxin-probes were present in sample 4B. This sample has also showed a high proportion of probes specific to clindamycin resistance genes (*ermF*). As for sample 7B, the presence of Porcine kobuvirus has been observed although with high background noise. The multiplex PCR reaction of these four samples confirmed the array results. However, it failed to detect Porcine kobuvirus in sample 8, which clearly appeared on the array. In this sample, a faint band is visible for *C. difficile* toxin A which presence was again indicated by the array. Moreover binary toxin amplification was not visible on the gel but seemed to be present in samples 8, C and 4B. The *C. difficile* toxin A gene might have been amplified by the PCR, however its molecular size made it undistinguishable from the 16S rRNA gene amplicon. The non-specific bands in the multiplex PCR possibly played a role in the high background noise observed in samples C and 4B, but this does not adequately explain the background noise in sample 7B.

A high prevalence of *C. difficile* has already been reported by many recent studies and it was believed to cause neonatal diarrhoea in piglets (Squire et al., 2013; Moono et al., 2016). Moreover, a highly virulent strain has been detected recently in piglets with diarrhoea (Wu et al., 2016). Porcine rotaviruses A are considered as very important epidemiologically and clinically in pigs and many authors described their presence in young piglets with diarrhoea (Chandler-Bostock et al., 2014; Lorenzetti et al., 2016; Theuns et al., 2016). Simultaneous detection of *C. difficile* and P. rotavirus has already been reported in diarrhoeic piglets (Farzan et al., 2013), others have found co-infection of pigs with *C. perfringens* and type A Porcine rotavirus (Cruz Junior et al., 2013).

Porcine kobuvirus has been isolated in both diarrhoeic and asymptomatic pigs, and the role of this pathogen in intestinal infections pathogenesis is still unclear. However, many authors have isolated this virus from pigs in European countries (Debast et al., 2009; Di Profio et al., 2013; Zhou et al., 2016). In China, Porcine kobuvirus was the first isolated causal viral agent of diarrhoea in pigs (Zhao et al., 2016).

Ideally, a successful hybridisation of a sample to an array would be a hybridisation where only the target(s) is/are present. However, this situation is less likely to be realistic in a clinical sample where a mixture of pathogens, commensal flora and host DNA are present. Extraction techniques are generally non-selective for microbial DNA. The detection of specific genes to targeted species is thus essential for the accuracy of the identification. The use of the whole sequence of the 16S rRNA as a target resulted in a high level of cross-

hybridisation between bacterial ribosomal 16S sequences, and this has eventually compelled us to remove the totality of ribosome gene-specific probes. Thus misidentifications could easily occur as there is only 0.17% of diversity among 16S rRNA genes in bacterial genomes (Stewart and Cavanaugh, 2007). Equally, poor quality sample where nucleic acid is degraded or in a minute amount can affect the amplification efficiency and consequently the microarray detection ability.

Despite the poor performance of the microarray in this study for the pathogen detection in 50% of bovine clinical samples, it performed successfully in identifying the reference strains. Moreover, the use of array hybridisation made the distinction between multiplex PCR products easier as it was not possible to differentiate between amplicons of similar size. It performed much better with the porcine samples. The reason for this is currently unclear but a closer examination of the probes and their performance leading to elimination of poor performing probes should improve this. This is validation by use, an approach which has been used by Dr. Malcom Banks at APHA, Weybridge in the past (pers. comm. to P. Barrow).

Probe design is an important step undertaken when developing a microarray as a diagnostic tool. Four probe design software (UPS, eArray, Picky and GoArray) were utilised based on published pathogen nucleotide sequences in GenBank. For each software, the same sequences were used, resulting in an output probe sequence set that relied on the strategy of the software used for selecting the best probes. Although statistically not significantly different,

probes designed with different software performed equally well. However, eArray, followed by UPS, yielded the higher detection rates compared to the other software. However, UPS, Picky and GoArray probes misidentified some of their targets; nonetheless they remained in a small proportion. Although GoArray and eArray 3' selection probes primarly for transcriptional analyses, which can lead to selective amplification of rRNA (Huang et al., 2014), in this study this was clearly not a problem.

The behaviour of the different probes cannot be totally predicted. Thermodynamic models might be good predictors of the binding behaviour of the probes. However, samples are generally sufficiently complex in structure prohibiting full modelling of the hybridisation behaviour of the probes. If parameters that include this particular complexity of the sample exist alongside hybridisation dynamics and all interactions that probes undergo with target and non-target sequences, their folding potential and formation of secondary structures, the modelling of probe hybridisation prediction, might then be fully applied.

Chapter 7. General Discussion and Conclusions

Several DNA-based microarray studies were carried out aiming at developing an array for the detection and identification of infectious microorganisms in clinical enteritis samples (Wang et al., 2002; Wattiau et al., 2008; Hauser et al., 2011; Jiang et al., 2010; 2011). In the field of infectious disease, microarrays have the potential to revolutionise research disease management (Bryant et al., 2004). The technology is regarded as highly reliable and offering a great rapidity of execution (Hong et al., 2004; Mao et al., 2008).

Intestinal disorders due to a microbial aetiology are the most important life threatening disease, particularly in young animals. Anamnesis and clinical signs are not sufficient for pathogen diagnosis. Molecular techniques are known to be reliable, precise and sensitive. However, the most challenging task in enteric infections diagnosis is the detection of multiple pathogens concomitantly present within a faecal sample.

The specific aim of this study was to design and develop and carry out preliminary evaluation of a DNA-based microarray for the detection of enteric pathogens in cattle and pigs. The microarrays developed in this study are considered the first to cover a broad range of pathogens causing enteric infections in cattle and pigs but not in humans. It is also the first study to assess the performance of two platforms with a common set of probes and with probes designed by four different software from common sequences on a single platform. Three main groups have developed a microarray detection for

enteric pathogens, they include Han et al. (2007) who used an in-house glass-spotted probes of 8 antigen O *Salmonella* serogroups genes. The second study by LeBlanc et al. (2009, 2010), showed the detection of four pestivirus species using microarray magnetic beads using array slides fabricated by GE Healthcare (LeBlanc et al., 2009). The same team carried out a microarray detection of pestiviruses using a suspension microarray coupled with real time PCR detection, using a Luminex platform (LeBlanc et al., 2010). In a third study (Jiang et al., 2010), the authors developed a microarray for the detection and genotyping of Porcine circovirus and differentiation between PCV-1 and PCV-2 in pigs; the microarrays were printed in-house on glass slides. All these studies were optimised to allow specific and sensitive detection.

The quality of the sample is an important parameter in pathogen identification, in particular when using molecular techniques that necessitate an irreproachable integrity of the DNA or RNA. Faeces remain one of the most difficult specimens for nucleic acid extraction due to the inhibitors contained in this material (Monteiro et al., 1997). Dependent on the host health condition and diet, faeces are a mixture of substances such as proteins, polysaccharide complexes, lipids, haemoglobin, bile salts, bilirubins, phenolic compounds and food degradation products (Nantavisai et al., 2007; Gonçalves et al., 2008).

It has been shown that incomplete removal of inhibitors reduces the sensitivity and specificity of molecular assays such as PCR or RT-PCR (Monteiro et al., 1997; Wilson, 1997; Das et al., 2009) by 1000-fold (Harris and Barletta, 2001; Ward and Wang, 2001). To avoid false negative results due to PCR inhibitors

present in faeces, it is essential to remove inhibitors from specimens by using reagents that degrade or absorb inhibitors as part of the extraction protocol. In this study, all techniques used preceding hybridisation were amplification-based using enzymes for the extension phase. Inhibitors can affect enzyme activity or bind directly to DNA preventing amplification. False negatives are often the result of a lack of amplification, and in this study, false negative results in the bovine samples might have been due to the utilisation of a kit (RTP Pathogen kit – Stratec Molecular) that offers the advantage of simultaneous extraction of pathogen DNA and RNA, but lacks a consideration of the specific nature of faecal material. However, it has been adapted by Fast-Track Diagnostics Ltd., Malta, for human stool (personal communication). Stratec uses a non-chaotropic technology unlike the majority of extraction kits that use guanidine to disrupt proteins, but which also may lead to nucleic acid denaturation. The benefit of the non-chaotropic salts is to ensure that enzymes in the mixture remain active and lytic. However, the drawback of this technology is undoubtedly its application for faecal specimens where nucleases are degrading DNA and RNA, and the problem is probably compounded when the DNA or RNA are single stranded, which is the case of most of diarrhoeic viral pathogens. The unsuitability of the Stratec kit for faecal sample nucleic acid extraction was questioned when microarray sensitivity, with spiked faeces experiments was assessed. Indeed, spiked bovine faecal samples with pelleted bacteria of descending dilutions of *E. coli* F5 were subjected to DNA extraction with Stratec followed with PCR detection using confirmed working primers that

are specific to the fimbrial subunit gene of *E. coli* F5. The PCR results (data not shown) were unanimously negative. This issue was overcome simply by changing the extraction kit to the QIAamp DNA Stool Mini Kit (Qiagen) which comprises a step where co-extracted PCR inhibitors with DNA were removed by adding an InhibitEX tablet (Included in the extraction kit) to the sample. After carrying out DNA extraction with the Qiagen kit, the F5 gene was amplified in dilutions -1, -2 and -3 (Fig. 6.18), with negative non-spiked faeces and negative control samples. This led to the conclusion that negative PCR results obtained using DNA extracted with the RTP kit might be an amplification failure due to PCR inhibitors, rather than an absence of the targeted gene in the sample.

Absence of pathogens in a diarrhoeic sample could also be the result of an excessively diluted specimen where the sample is watery; a calf with diarrhoea could lose up to seven litres of water per day (Smith and Berchtold, 2014). On the other hand, in this study extracted nucleic acid was transported and kept at room temperature in a preservative substance (DNAStable and RNAStable, Biomatrica). Most of studies that carried out testing of the stability of nucleic acid subjected to accelerated ageing involved DNA and the majority showed that DNAStable was effective for the preservation of DNA at room temperature (Lee et al., 2012; Ivanova and Kuzmina, 2013; Howlett et al., 2014). However, long fragments of DNA (500-1000 bp) are more susceptible to degradation (Howlett et al., 2014). In other studies, preservation of RNA in RNAStable showed nearly the same preservation efficiency as freezing samples for RNA

extraction from human blood (Seelenfreund et al., 2014) and total RNA from human cells (Stevenson et al., 2015). However, no study has evaluated microbial RNA and DNA stabilisation extracted from a mono- or poly-infected specimen at room temperature. Hernandez et al. (2009) reported that the drying step in the sample preparation is critical for the thermic stability of the RNA.

It must also be remembered that diarrhoea in calves can also have a nutritional origin not involving infectious agents (Wenge et al., 2014) which would also generate negative signals.

Molecular techniques such as PCR have been used extensively for microbial diagnosis in clinical samples. With the limitations of PCR and multiplex PCR regarding the number of targets, microarray is one of the methods of choice for multiple detection. Indeed, a single experiment allows the simultaneous detection of a wide variety of infectious disease markers including almost the whole genome of viruses, virulence factors for bacteria and parasites, antibiotic and drug resistance markers.

Two different amplification strategies were used in this study, random and sequence-specific. The combined use of specific PCR followed by random amplification allows the identification of specific targets in addition to any other non-targeted sequences whereas the use of random amplification reduces the likelihood of obtaining sufficient quantities of the specific target that might be present to be detectable on the array.

These two amplification approaches were compared using the ArrayTube platform. Array hybridisation with reference strains resulted in more specific detection following random amplification compared to the sequence-specific amplification. However, signal intensities were higher when specific amplification was performed. Surprisingly, the random amplification failed to identify the viruses in porcine clinical samples, which were shown to be positive to Porcine rotavirus and Porcine kobuvirus by PCR.

Random amplification has been described as simple, inexpensive, rapid and useful technique for typing low number of microbial strains (Gravesen et al., 2000) and several authors reported an increased analytical sensitivity of real time quantitative reverse transcription when random primers were used compared to gene specific primers (Ståhlberg et al., 2004; Nardon et al., 2009). Its main advantage is that it does not require a priori knowledge of the organism genome for its identification (Vora et al., 2004). However, non-specific target sequences have the same chances to be amplified than the target sequence of interest, the former including host DNA or commensal DNA present in a complex sample like faeces. This can undeniably lead to a matrix of amplified targets that could compete against binding sites during hybridisation. Wang et al. (2010) showed that this method was less sensitive than amplification via multiplex PCR, unless when pure cultured bacteria were used at the required concentration, which is probably the case in this study where reference strains were hybridised.

Although sequence-specific amplification prior to array hybridisation was demonstrated to be specific and sensitive (Vora et al., 2004; McLoughlin, 2011), in the absence of additional sequencing, it does not lead to the discovery of novel species or the detection of variant strains of known species (McLoughlin, 2011).

It was clear from the work with the Alere array that random amplification is the ideal option for diagnosis and surveillance. However, considerable technical improvements are required before this can become a reality and this was not explored with the Agilent array. If this platform is used for confirmation and other more targeted studies but utilising the wider spread of probes, then specific amplification may be more appropriate for the slide array.

Further work should be directed towards improving technologies which will allow specific removal of host DNA. In clinical samples this is always likely to be a feature, less so, with enteritis which involves fluid secretion, as induced by ETEC strains, in comparison with those caused by for example, *Salmonella,* where considerable intestinal tissue destruction may follow infection. However, it is appreciated that removal of host NA could also bring down pathogen nucleic acid affecting sensitivity.

It is relevant after this study to explore the role of microarrays in diagnosis and surveillance given that although, in theory, they can resolve mixed infections resulting from involvement of a number of pathogens, in this study, specific amplification was used and samples were pre-analysed by PCR including multiplex PCR, in this case, to ensure that the relevant DNA species were

present prior to application to the array. However, if this is rolled out into a field situation it is pertinent to discuss the value of the array if equally valid results are obtainable by multiplex PCR. Although the sensitivity of the PCRs done here left something to be desired, the array appeared nevertheless to be of greater sensitivity than the straight PCRs. From this point of view further exploration of the role and strength/weakness of random versus specific priming would be of value.

Hybridisation to the array clearly showed cross-hybridisation with almost all probes corresponding to the 16S rRNA of different species of bacteria. The 16S ribosomal RNA (rRNA) is a genomic marker that has been utilised extensively for bacterial detection and identification by PCR in a range of samples, from environmental to clinical and from commensal to pathogenic flora. The bacterial 16S rRNA has conserved and variable regions (Van de Peer et al., 1996) that can be used successfully in identifying related bacterial species (Bertilsson et al., 2002; Becker et al., 2004). Nonetheless, strain discrimination is often difficult when diagnosis is based on 16S rRNA detection only. For instance, the 16S rRNA of pathogenic *E. coli* (O157:H7) is indistinguishable from commensal *E. coli* (K12) (Weinstock, 2012). In a recent study, the utilisation of meta-taxonomics (16S rRNA marker gene sequencing) was able to identify bacteria only at the species level (Hilton et al., 2016). In fact, two species are considered identical when their respective 16S rRNA sequences are at least 97% identical (Stackebrandt and Goebel, 1994). Recently, a new threshold of 98.65% has been proposed based on the sequencing results of 6787 genome

sequences (Kim et al., 2014). In this study bacterial reference species were identified using 16S rRNA specific primers. For this main reason, 16S sequencing, is being gradually abandoned in favour of whole genome sequencing, where thousands of microrganisms are identified through millions of their specific genes. This has predominantly been applied to study the gut microbiota in humans, as it also allows detection of members of different kingdoms.

In terms of disease detection, the composition and the abundance of specific markers in the gut metagenome, have revealed differences between healthy and ill individuals. In animals, and pigs in particular, sequencing of the total extracted DNA was used to understand the association between the intestinal microbiome and a particular condition such as stress, diet and antibiotics. Also few studies have estimated the variation of the bacterial and viral proportions in healthy versus diarrhoeic pigs.

In this study, the whole sequence of 16S rRNA, which is approximatively 1.5 kb, was targeted entirely using primers that were designed from the 3' and 5' conserved regions of this gene. However, to prevent massive cross-hybridisation events, all signal intensities results of ribosomal probes were removed from the data, which allowed consideration of virulence gene and antibiotic gene specific probes only for the characterisation of bacterial species.

Hybridisation signals depend highly on experimental parameters such as probe length and density (Relógio et al., 2002; Chou et al., 2004; Jayaraman et al.,

2007; Singh et al., 2009), temperature (Taroncher-Oldenburg et al., 2003; Lee et al., 2004; Poulsen et al., 2008), time (Dorris et al., 2003; Sartor et al., 2004), ionic strength of the hybridisation buffer (Gong and Levicky, 2008), washing stringency (Drobyshev et al., 2003; Li et al., 2004) and other reagents (Ku et al., 2004) included in the hybridisation solution. Hybridisation temperature and salt concentration, which together define the stringency, are the important parameters to consider. Extremely high and low stringency conditions lead to washing-off the specific signal and to non-specific binding, respectively. Stringency levels must be high enough to prevent non-specific binding, but not so high that specific signal is removed by the washings (Korkola et al., 2003).

It is clear from the discussion sections in chapters 5 and 6 that probe design is a crucial step in genotyping microarray development; its efficiency determines the specificity of the probes and impacts the microarray performance. In this study, design of long probes was chosen as they constitute a balance between specificity and sensitivity, indeed long probes have higher sensitivity (Relógio et al., 2002; Fenart et al., 2013) and less specificity (Harrington et al., 2008) than short probes. They also tolerate more mismatches during the formation of duplexes with targets, which is a critical limitation of this approach when using genus- and family-specific probes such as the 16S rRNA or 18S rRNA. Thus the design of probes from these particular genes should present a reasonable diversity for improved discriminatory abilities. The choice of the region of the genome for probe design should allow a specific identification for unambiguous

results; this can be achieved by using species/strain specific genes such as virulence or antibiotic resistance genes.

In this study it was challenging to design probes specific to closely related species (Enterobacteria for instance) as it has resulted in a high level of cross-reactivity. To avoid cross-reactivity of the probes with non-targets that might be present in the sample, it is important to perform an *in silico* specificity test using the NCBI BLAST programme, independently of the design software that has a built-in feature to test the probes via an external link to BLAST. Probes should then be categorised based on their uniqueness. Further to this, probes with similar melting temperatures should be selected to be able to control the stringency of hybridisation (Barra et al., 2013).

Thermodynamic calculations should also allow selecting probes that are less prone to secondary structures formation and those which show a perfect match with their respective targets, favouring duplexes with the lowest free energies.

As with all diagnostic techniques, microarrays have weaknesses. Potential difficulties in the reproducibility of microarrays have been reported due to technical reasons such as slide heterogeneity, printer-pin variation, and spot size differences (Bryant et al., 2004), presence or absence of attachment spacers (Loy et al., 2002) and batch effects which refers to a systematic error resulting from microarray processing at one site over a short period of time (Chen et al., 2011).

In this study, the 15993 designed probes covered 54 pathogen species/serotypes. Virus probes (n= 201) designed with UPS software were spotted onto Alere microarray platform. All other probes, including the Alere probes were printed onto 4x44k Agilent platform. Probes for Agilent microarray platforms were designed using the four software. In terms of software design-based comparison, which was based on the hybridisation results of reference strains, although not statistically different, the best performing probes were eArray probes followed by UPS, GoArray and Picky. Equally with regard to falsely positive probes, UPS, Picky and GoArray registered the highest number of misidentified probes. It is important to point out that the number of probes per pathogen and per software should be taken into consideration. Furthermore, as eArray was the probe design software of Agilent, it was expected that these probes would achieve the highest performance among the other software.

The evaluation process included specificity and sensitivity testing that will allow a further reduction in the number of designed probe via eliminating probes showing poor or no signal during array hybridisation.

In this study the ArrayTube platform showed a relatively good level of specificity with five out of eight reference viral species identified by the array. Additionally, the hybridisation of multiplexed TGEV and PCV-2 with three species of bacteria (Multiplex_1), and PEDV and Porcine rotavirus A with two bacteria and one parasite (Multiplex_2) resulted in a successful detection of TGEV, PCV-2 and PEDV. A relatively acceptable sensitivity was achieved by the

ArrayTube microarray for the detection of PCV-2 and TGEV, with a 2.06 x 10^9 and 4.84 x 10^5 copy number detected respectively. It is likely that the number of virus particles and therefore copies would exceed this in diarrhoea. However, issues of sensitivity of such assays and how the pathogen count (copy number) relates to the production of disease is highly relevant since not only can host genetic background affect susceptibility and therefore the number of virus particles likely to be present but the presence of pathogens in a healthy animal must also be considered. The numbers of individual pathogen cells required to produce disease may also vary depending on whether single or mixed infections are involved. However, these are more philosophical discussions which must be addressed once the more technical aspects of array-based diagnosis are dealt with.

To the best of our knowledge, this is the first study where an Agilent microarray was used to detect intestinal infection in cattle and pigs. Hybridisation results of four viral species to the microarray showed that TGEV, PEDV, PCV-2 were detected, whereas only seven probes were positive for Porcine rotavirus A, which also showed no hybridisation with the ArrayTube platform. Among hybridised reference strains of bacteria and parasites to the Agilent platform, *C. difficile*, *C. jejuni*, *E. coli* F4, *E. coli* F5 and *B. pilosicoli*, *Salmonella* and *E. acervulina* were correctly identified. Furthermore, when two multiplex PCR targets (Five pathogens each) were hybridised to the Agilent microarray, the ten pathogens were identified accurately. The sensitivity of the array was on occasions poor and reflected the PCR results which requires further

investigation to determine whether this was the result of a technical shortcoming.

Microarrays are reported to lack reproducibility. The requirements due to manufacturing, hybridisation and labelling, data acquisition and normalisation methods have led to discrepancies in array results. In this study PCV detection of diluted DNA indicated a reliable reproducibility compared to TGEV. The reproducibility reflects the quality and printing consistency of the array. The fact that PCV indicated a reasonable good reproducibility, while TGEV did not, suggest that further dilutions of TGEV DNA would be needed to clarify this point.

With clinical samples, the ArrayTube microarray was able to detect Porcine kobuvirus, but failed to identify Porcine rotavirus A in the same sample. Similarly in the same sample, these two viruses were detected by the Agilent microarray, alongside *C. difficile*, *C. difficile* toxin A and binary toxin. These findings appeared to show the presence of mixed infection in piglets, represented by two viruses and one bacterium with two toxins. Co-infected piglets with *C. difficile* and P. rotavirus A were reported (Chan et al., 2013), and no such co-infection of pigs with three pathogens has been reported in the literature.

While microarray represents a useful technology for use in pathogen detection, particularly in multi-aetiological infectious disease such as enteritis, in this study one of the major limitations that was faced consisted firstly of the high level of cross-hybridisation between 16S rRNA and 18S rRNA probes with

bacteria and parasites respectively. Secondly, the probe design was definitive and not easily updated, unless more designed probes are included onto the platform and new microarrays are produced. This is problematic and would be costly to keep-up-to date in terms of the involvement of infectious pathogens or potentially new discovered pathogens that play a minor or major role in the disease dynamics. For instance, it was recently demonstrated that a new neonatal diarrhoea caused by *Enterococcus hirae* (Larsson et al., 2014, 2015; Jonach et al., 2014; Hermann-Bank et al., 2015) - has emerged in the porcine population in Europe. This bacterium was considered as a prevalent causative agent of enteritis in pigs in this study shortly after the gene database was constructed and the probes designed in this study. A recently discovered enteric virus consists of a new member of the *Coronaviridae* family, porcine deltavirus, that has been detected in pigs with diarrhoea (Sinha et al., 2015; McCluskey et al., 2016).

Probes designed with UPS (and spotted in Alere microarray platform ArrayTube) were tested thermodynamically. Among 13 porcine virus probes, nine probes showed comparable high signal intensities in both ArrayTube and Agilent microarray platforms. The general signal intensity trend showed proportionality between the two platforms.

The folding minimal free energy predictions varied between +0.46 and -7.16 kcal/mol with nine probes recording a free energy below -4. When the free energy tends towards low negative values, probes have an increased likelihood to form stable secondary structures (interior loop, hairpin and bulges mainly).

Very few probes performed well, in terms of hybridisation, even though the folding free energies were low. However, the highest signal intensities were registered by probes with free energies tending towards 0. This might also indicate that free energy variation should be relatively high to impact the hybridisation efficiency, for instance a probe showed a free energy of -7.16 Kcal/mol but, nevertheless, recorded a relatively high signal intensity. Moreover, it is possible that the probe was sufficiently long to allow interactions involving self-folding to form a secondary structure and being simultaneously fully or partially hybridised to yield a detectable signal. A higher number of probes should be assessed thermodynamically for a better interpretation, as the actual data might seem subjective due to the low number of probes compared.

As part of the current study, comparison of *in silico* hybridisation prediction and wet/experimental hybridisation testing using sequences from some of the viruses indicated that there was no agreement between the two methods.

Both laboratory hybridisation assays and computer-based thermodynamics should allow a better selection of the best probes for microarray development, although more hybridisations and more probe and probe-target duplex stability predictions are clearly necessary. Indeed, it is practically impossible to test individually all 15993 designed probes, although *in silico* specificity, secondary structure prediction of the probes, coupled with duplex formation prediction is a perfectly achievable task. Although computational modelling can certainly not be comparable to laboratory experimental assays it has the

potential to assist in evaluating the probes and the platforms in terms of hybridisation efficiency.

In any technological development work, experimental optimisation is an important step to undertake before performing the assay. Many authors (Bowtell, 1999; Peplies et al., 2003; Rouse et al., 2008) stressed the vital necessity of optimisation of experiment parameters, thus resulting in a decisive outcome of such approach. In this study, no excessive optimisations of the microarray hybridisation were performed. In-house and commercial protocols were used by this research group at Nottingham Veterinary School. The results obtained in this study were achieved without optimisation, which means that the performance of the microarray technique components (specificity and sensitivity) could undoubtedly be improved further. Optimisations commonly involve improvements of each step performed, including probe design (highly species specific, design of sets of probes with approximatively the same melting temperature, high GC content in the central area of the probe) and hybridisation temperature optimisation by attempting touch-down hybridisation.

The speed with which sequencing and next generation sequencing is evolving nowadays, has resulted in a similar development and improvement of the sensitivity of microarray platforms in comparison with sequencing platforms. Much effort has been concentrated on labelling strategies, where fluorescent semi-conductor quantum dots (QDs) appear to be increasingly used as they are 20 times brighter and 100 times more stable than organic dyes (Chan and Nie,

1998). Also metal colloid labels using principally gold and silver have been used in microarray and detected with optical and electrical devices (Francois et al., 2003; Zhao et al., 2010). Some recent research studies have focused on a reduced number of sample preparation steps involving label free detection, based only on accumulated negative charges during hybridisation, which are subsequently measured by electrochemical impedance spectroscopy (Henihan et al., 2016). Others have integrated the microarray platform in a compact, portable microfluidic device with label free biosensor (Sun, 2015) that can also be utilised for point of care testing (sample-to-result). However, none has been developed for stool/faecal samples. This is clearly an opportunity for further translational developments which would be a natural outcome of the present work and towards which further work will be directed. In terms of a universal microbial detection solution, Axiom microbiome, a high density microarray genotyping platform from Affymetrix, seems to offer a good alternative for microbial identification in a multitude of biological samples such as urine, stool, tonsil and serum.

For future array work, the 16S rRNA probes should either be removed from the platform or combined with additional discriminatory probes from variable region of the 16S rRNA and other pathogen-specific sequences on the respective genomes. Furthermore, probes that successfully pass the BLAST test, thermodynamics and the melting temperature filters would be spotted on a future version of the array. We would recommend exploiting the flexibility of

Agilent microarray platform to accommodate and test emerging sequences as a single array which would significantly reduce the cost.

The issue of cut-off values to determine whether samples were positive was also a major issue during this study. Many probes were clearly cross-hybridising and would be de-selected for future work. Additional approaches were used in this study consisting of the identification of probes that gave signals higher than the mean added to three times the standard deviation of the background signals. In this study the cut-offs were valid as the PCR products of the clinical samples were hybridised to the array, meaning that the probes giving valid signals were expected to belong to the pathogen that was detected by PCR.

There are other approaches which could be considered and related to the number of probes designed and utilised for each pathogen. Problems sometimes occur with arrays used both for diagnosis/detection and also for gene expression analysis, and that is that within any cluster of genes or related probes, the interpretation of the occurrence of a positive result arising from just a small proportion of these genes/probes requires considerable thought. Thus, there is an approach whereby the occurrence of positive signals from a high proportion of probes for any pathogen might be considered as a positive for that pathogen. This then leads to the question of what proportion should be used and this also presents problems for small genome pathogens such as viruses compared with bacteria. However, this is worth consideration for future work and analysis.

More testing using a larger panel of reference strains will be necessary to facilitate refinement of a probe set which produces discriminatory results of adequate sensitivity. Likewise, more clinical samples, confirmed to have an infectious origin should be tested with the microarray for the identification of the aetiological agent(s).

The investigation carried out in this study would ideally also form part of the pipeline for future development of a portable all in one device for enteric pathogen detection.

References

Abe, M., Ito, N., Sakai, K., Kaku, Y., Oba, M., Nishimura, M., Kurane, I., Saijo, M., Morikawa, S., Sugiyama, M., Mizutani, T., 2011. A novel sapelovirus-like virus isolation from wild boar. Virus Genes 43, 243–248.

Abraham, G., Roeder, P.L., Zewdu, R., 1992. Agents associated with neonatal diarrhoea in Ethiopian dairy calves. Trop. Anim. Health Prod. 24, 74–80.

Achá, S.J., Kühn, I., Jonsson, P., Mbazima, G., Katouli, M., Möllby, R., 2004. Studies on calf diarrhoea in Mozambique: prevalence of bacterial pathogens. Acta Vet. Scand. 45, 27–36.

Achacha, M., Messier, S., Mittal, K.R., 1996. Development of an experimental model allowing discrimination between virulent and avirulent isolates of Serpulina (Treponema) hyodysenteriae. Can. J. Vet. Res. = Rev. Can. Rech. vétérinaire 60, 45–9.

Adesiyun, a a, Kaminjolo, J.S., Loregnard, R., Kitson-Piggott, W., 1992. Campylobacter infections in calves, piglets, lambs and kids in Trinidad. Br. Vet. J. 148, 547–56.

Ahn, B.C., Walz, P.H., Kennedy, G.A., Kapil, S., 2005. Biotype, Genotype, and Clinical Presentation Associated With Bovine Viral Diarrhea Virus (BVDV) Isolates From Cattle. Intern J Appl Res Vet Med 3, 319–325.

Akam, A., Khelef, D., Kaidi, R., Rahal, K., Tali-Maamar, H., Yabrir, B., Laoun, A., Mostfaoui, A., Boutaiba, S., Cozma, V., 2011. The Frequency of the Shedding of Cryptosporidium parvum, F5 Escherichia coli, Rotavirus, Coronavirus and Salmonella spp. in Young Dairy Calves in Mitidja Area (Algeria). Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca. Vet. Med. 2.

Albini, S., Brodard, I., Jaussi, a, Wollschlaeger, N., Frey, J., Miserez, R., Abril, C., 2008. Real-time multiplex PCR assays for reliable detection of Clostridium perfringens toxin genes in animal isolates. Vet. Microbiol. 127, 179–85.

Alexandre, I., Hamels, S., Dufour, S., Collet, J., Zammatteo, N., De Longueville, F., Gala, J.-L., Remacle, J., 2001. Colorimetric Silver Detection of DNA Microarrays. Anal. Biochem. 295, 1–8.

Alfieri, A.F., Alfieri, A.A., Barreiros, M.A.B., Leite, J.P.G., Richtzenhain, L.J., 2004. G and P genotypes of group A rotavirus strains circulating in calves in Brazil, 1996-1999. Vet. Microbiol. 99, 167–73.

Alkan, F., Karayel, I., Catella, C., Bodnar, L., Lanave, G., Bányai, K., Di Martino, B., Decaro, N., Buonavoglia, C., Martella, V., 2015. Identification of a Bovine enteric Calicivirus, Kırklareli virus, distantly related to Neboviruses, in calves with

enteritis in Turkey. J Clin Microbiol. 53(11): 3614–3617.

Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. 12, 3–14.

Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Botner, A., McCullough, K., Nauwynck, H., Kennedy, S., Meehan, B., Charreyre, C., 2004. PMWS: experimental model and co-infections. Vet. Microbiol. 98, 165–168.

Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J. a., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of Porcine Circovirus-like Viruses from Pigs with a Wasting Disease in the USA and Europe. J. Vet. Diagnostic Investig. 10, 3–10.

Alonso-Padilla, J., Pignatelli, J., Simon-Grifé, M., Plazuelo, S., Casal, J., Rodríguez, D., 2012. Seroprevalence of porcine torovirus (PToV) in Spanish farms. BMC Res. Notes 5, 675.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Álvarez-Fernández, R. 2013. Chapter One – Explanatory Chapter: PCR Primer Design. Methods Enzymol. 529: 1-21.

Alwine, J.C., Kemp, D.J., Stark, G.R., 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Biochemistry 74, 5350–5354.

Anbalagan, S., Peterson, J., Wassman, B., Elston, J., Schwartz, K., 2014. Genome sequence of torovirus identified from a pig with porcine epidemic diarrhea virus from the United States. Genome Announc. 2.

Andrade, G.I., Coura, F.M., Santos, E.L.S., Ferreira, M.G., Galinari, G.C.F., Facury Filho, E.J., de Carvalho, A.U., Lage, A.P., Heinemann, M.B., 2012. Identification of virulence factors by multiplex PCR in Escherichia coli isolated from calves in Minas Gerais, Brazil. Trop. Anim. Health Prod. 44, 1783–1790.

Andress, C.E., Barnum, D.A., Thomson, R.G., 1968. Pathogenicity of vibrio coli for swine. I. Experimental infection of gnotobiotic pigs with vibrio coli. Can. J. Comp. Med. Rev. Can. médecine Comp. 32, 522–8.

Angus, K.W., 1983. Cryptosporidiosis in man, domestic animals and birds: a review. J. R. Soc. Med. 76, 62–70.

Anjum, M.F., Mafura, M., Slickers, P., Ballmer, K., Kuhnert, P., Woodward, M.J., Ehricht, R., 2007. Pathotyping Escherichia coli by using miniaturized DNA microarrays. Appl. Environ. Microbiol. 73, 5692–7.

Arcellana-Panlilio M. and Robbins S.M. 2002. Cutting edge technology I- Global gene

expression profiling using DNA microarrays. Am. J. Physiol. Gastrointestinal Liver Physiol. 282: G397-G402.

Attoui, H., Mohd Jaafar, F., Belhouchet, M., Biagini, P., Cantaloube, J.F., De Micco, P., De Lamballerie, X., 2005. Expansion of family Reoviridae to include nine-segmented dsRNA viruses: Isolation and characterization of a new virus designated aedes pseudoscutellaris reovirus assigned to a proposed genus (Dinovernavirus). Virology 343, 212–223.

Baccaro, M.R., Moreno, A.M., Shinya, L.T., Dotto, D.S., Medicina, F. De, Paulo, U.D.S., Paulo, S., 2003. Identification of bacterial agents of enteric diseases by multiplex PCR in growing-finishing pigs. Brazilian J. Microbiol. 34, 225–229.

Baker, J.C., 1990. Clinical aspects of bovine virus diarrhoea virus infection. Rev. Sci. Tech. 9, 25–41.

Ballarini, A., Segata, N., Huttenhower, C., Jousson, O., 2013. Simultaneous quantification of multiple bacteria by the BactoChip microarray designed to target species-specific marker genes. PLoS One 8, e55764.

Bank-Wolf, B.R., König, M., Thiel, H.-J., 2010. Zoonotic aspects of infections with noroviruses and sapoviruses. Vet. Microbiol. 140, 204–12.

Bányai, K., László, B., Duque, J., Nelson, E.A.S., Gentsch, J.R., 2012. Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: Insights for understanding the impact of rotavirus vaccination programs. Vaccine 30, A122–A130.

Barketi-Klai, A., Hoys, S., Lambert-Bordes, S., Collignon, A., Kansau, I., 2011. Role of fibronectin-binding protein A in Clostridium difficile intestinal colonization. J. Med. Microbiol. 60, 1155–1161.

Barlag, B., Hensel, M., 2015. The giant adhesin SiiE of salmonella enterica. Molecules.

Barnes, M.A., Wright, R.E., Bodine, A.B., Alberty, C.F., 1982. Frequency of bluetongue and bovine parvovirus infection in cattle in South Carolina dairy herds. Am. J. Vet. Res. 43, 1078–1080.

Barra, L., Ruggiero, M.V., Sarno, D., Montresor, M., Kooistra, W.H.C.F., 2013. Strengths and weaknesses of microarray approaches to detect Pseudo-nitzschia species in the field. Environ. Sci. Pollut. Res. 20, 6705–6718.

Barrow, P., Duchet-Suchaux, M., 1997. Salmonella carriage and the carrier state. In: Salmonella and Salmonellosis 97 Proceedings. Zoopôle, Ploufragan, France, pp. 241–250.

Barry, A.F., Ribeiro, J., Alfieri, A.A.F.A.A., van der Poel, W.H.M.M., Alfieri, A.A.F.A.A., 2011. First detection of kobuvirus in farm animals in Brazil and the Netherlands.

Infect. Genet. Evol. 11, 1811–4.

Bartels, C.J.M., Holzhauer, M., Jorritsma, R., Swart, W.A.J.M., Lam, T.J.G.M., 2010. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Prev. Vet. Med. 93, 162–169.

Barth, S., Gömmel, M., Baljer, G., Herbst, W., 2012. Demonstration of genes encoding virulence and virulence life-style factors in Brachyspira spp. isolates from pigs. Vet. Microbiol. 155, 438–43.

Baskerville, A., McFerran, J.B., Connor, T., 1971. The pathology of experimental infection of pigs with type I reovirus of porcine origin. Res. Vet. Sci. 12, 172–4.

Baxi, M., McRae, D., Baxi, S., Greiser-Wilke, I., Vilcek, S., Amoako, K., Deregt, D., 2006. A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. Vet. Microbiol. 116, 37–44.

Beards, G.M., Hall, C., Green, J., Flewett, T.H., Lamouliatte, F., Du Pasquier, P., 1984. An enveloped virus in stools of children and adults with gastroenteritis that resembles the Breda virus of calves. Lancet (London, England) 1, 1050–2.

Becher, P., Orlich, M., Shannon, A.D., Horner, G., Ko$ Nig, M., Rgen Thiel, H.-J., 1997. Printed in Great Britain Phylogenetic analysis of pestiviruses from domestic and wild ruminants. J. Gen. Virol. 78, 1357–1366.

Becker, A., Reith, A., Napiwotzki, J., Kadenbach, B., 1996. A Quantitative Method of Determining Initial Amounts of DNA by Polymerase Chain Reaction Cycle Titration Using Digital Imaging and a Novel DNA Stain. Anal. Biochem. 237, 204–207.

Becker, K., Harmsen, D., Mellmann, A., Meier, C., Schumann, P., Peters, G., von Eiff, C., 2004. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of Staphylococcus species. J. Clin. Microbiol. 42, 4988–95.

Beld, M., Minnaar, R., Weel, J., Sol, C., Damen, M., van der Avoort, H., Wertheim-van Dillen, P., van Breda, A., Boom, R., 2004. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. J. Clin. Microbiol. 42, 3059–64.

Belliot G., Sosnovtsev S.V., Mitra T., Hammer C., Garfield M., G.K.Y., 2003. In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. J. Virol. 77, 10957 –10974.

Bellows, R. a, Patterson, D.J., Burfening, P.J., Phelps, D. a, 1987. Occurrence of neonatal and postnatal mortality in range beef cattle. II. Factors contributing to calf death. Theriogenology 28, 573–586.

Beloeil, P., Fravalo, P., Fablet, C., Jolly, J.-P., Eveno, E., Hascoet, Y., Chauvin, C., Salvat, G., Madec, F., 2004. Risk factors for Salmonella enterica subsp. enterica shedding by market-age pigs in French farrow-to-finish herds. Prev. Vet. Med. 63, 103–20.

Bendali, F., Bichet, H., Schelcher, F., Sanaa, M., 1999. Pattern of diarrhoea in newborn beef calves in south-west France. Vet. Res. 30, 61–74.

Bergeron, J., Menezes, J., Tijssen, P., 1993. Genomic Organization and Mapping of Transcription and Translation Products of the NADL-2 Strain of Porcine Parvovirus. Virology 197, 86–98.

Bertilsson, S., Cavanaugh, C.M., Polz, M.F., 2002. Sequencing-independent method to generate oligonucleotide probes targeting a variable region in bacterial 16S rRNA by PCR with detachable primers. Appl. Environ. Microbiol. 68, 6077–6086.

Binder, H., Kirsten, T., Loeffler, M., Stadler, P.F., 2004. Sensitivity of Microarray Oligonucleotide Probes: Variability and Effect of Base Composition. J. Phys. Chem. B 108, 18003–18014.

Blanchard, P., Mahé, D., Cariolet, R., Truong, C., Le Dimna, M., Arnauld, C., Rose, N., Eveno, E., Albina, E., Madec, F., Jestin, A., 2003. An ORF2 protein-based ELISA for porcine circovirus type 2 antibodies in post-weaning multisystemic wasting syndrome. Vet. Microbiol. 94, 183–194.

Blanchard, P.C., 2012. Diagnostics of dairy and beef cattle diarrhea. Vet. Clin. North Am. Food Anim. Pract. 28, 443–64.

Blomström, A.-L., Belák, S., Fossum, C., Fuxler, L., Wallgren, P., Berg, M., 2010. Studies of porcine circovirus type 2, porcine boca-like virus and torque teno virus indicate the presence of multiple viral infections in postweaning multisystemic wasting syndrome pigs. Virus Res. 152, 59–64.

Blomström, A.L., Belák, S., Fossum, C., McKillen, J., Allan, G., Wallgren, P., Berg, M., 2009. Detection of a novel porcine boca-like virus in the background of porcine circovirus type 2 induced postweaning multisystemic wasting syndrome. Virus Res. 146, 125–129.

Blomström, A.-L., Ståhl, K., Okurut, A.R., Masembe, C., Berg, M., 2013. Genetic characterisation of a porcine bocavirus detected in domestic pigs in Uganda. Virus Genes 47, 370–3.

Boesen, H.T., Jensen, T.K., Schmidt, A.S., Jensen, B.B., Jensen, S.M., Møller, K., 2004. The influence of diet on Lawsonia intracellularis colonization in pigs upon experimental challenge. Vet. Microbiol. 103, 35–45.

Bohlander, S.K., Espinosa, R., Le Beau, M.M., Rowley, J.D., D??az, M.O., 1992. A method for the rapid sequence-independent amplification of microdissected chromosomal material. Genomics 13, 1322–1324.

Boileau, M.J., Kapil, S., 2010. Bovine coronavirus associated syndromes. Vet. Clin. North Am. Food Anim. Pract. 26, 123–46, table of contents.

Bonardi, S., Paris, A., Bacci, C., D'Incau, M., Ferroni, L., Brindani, F., 2007. Detection and Characterization of Yersinia enterocolitica from Pigs and Cattle. Vet. Res. Commun. 31, 347–350.

Booth, R.E., Thomas, C.J., El-Attar, L.M., Gunn, G., Brownlie, J., 2013. A phylogenetic analysis of Bovine Viral Diarrhoea Virus (BVDV) isolates from six different regions of the UK and links to animal movement data. Vet. Res. 44.

Bora, D., Bhattacharyya, D., Barman, N., Dutta, L., 2009. Rotavirus excretion by diarrheic and healthy pigs in organized herds in Assam. Anim. Sci. Report. 2, 57–61.

Borer, P.N., Dengler, B., Tinoco, I., Uhlenbeck, O.C., 1974. Stability of ribonucleic acid double-stranded helices. J. Mol. Biol. 86, 843–853.

Borewicz, K.A., Kim, H.B., Singer, R.S., Gebhart, C.J., Sreevatsan, S., Johnson, T., Isaacson, R.E., 2015. Changes in the Porcine Intestinal Microbiome in Response to Infection with Salmonella enterica and Lawsonia intracellularis. PLoS One 10, e0139106.

Boros, A, Nemes, C., Pankovics, P., B??r??, H., Kapusinszky, B., Delwart, E., Reuter, G., 2012a. Characterization of a novel porcine enterovirus in wild boars in Hungary. Arch. Virol. 157, 981–986.

Boros, A, Nemes, C., Pankovics, P., Kapusinszky, B., Delwart, E., Reuter, G., 2012b. Porcine teschovirus in wild boars in Hungary. Arch. Virol. 157, 1573–1578.

Boros, A., Pankovics, P., Reuter, G., 2011. Characterization of a novel porcine enterovirus in domestic pig in Hungary. Infect. Genet. Evol. 11, 1096–1102.

Bowtell, D.D., 1999. Options available--from start to finish--for obtaining expression data by microarray. Nat. Genet. 21, 25–32.

Bratz, K., Bücker, R., Gölz, G., Zakrzewski, S.S., Janczyk, P., Nöckler, K., Alter, T., 2013. Experimental infection of weaned piglets with Campylobacter coli – Excretion and translocation in a pig colonisation trial. Vet. Microbiol. 162, 136–143.

Brenner, D., 1992. Additional genera of the Enterobacteriaceae. In: Balows, A., Tru¨per, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. New York, NY, Springer, pp. 2922–2937.

Breslauer, K.J., Frank, R., Blöcker, H., Marky, L.A., 1986. Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. U. S. A. 83, 3746–50.

Brian, D.A., Baric, R.S., 2005. Coronavirus Genome Structure and Replication. Curr.

Top. Microbiol. Immunol. 287, 1–30.

Bridger J.C., 1990. Small viruses associated with gastroenteritis in animals. In: Saif, L.J., Theil, K.W. (Ed.), Viral Diarrheas of Man and Animals. Boca Raton, pp. 161–182.

Bridger, J., Woode, G., Meyling, A., 1978. Isolation of coronaviruses from neonatal calf diarrhoea in Great Britain and Denmark. Vet. Microbiol.

Brown, D.W., Beards, G.M., Flewett, T.H., 1987. Detection of Breda virus antigen and antibody in humans and animals by enzyme immunoassay. J. Clin. Microbiol. 25, 637–40.

Brownlie, J., 1990. The pathogenesis of bovine virus diarrhoea virus infections. Rev. Sci. Tech. 9, 43–59.

Brunborg, I.M., Moldal, T., Jonassen, C.M., 2004. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. J. Virol. Methods 122, 171–178.

Bryant, P.A., Venter, D., Robins-Browne, R., Curtis, N., 2004. Chips with everything: DNA microarrays in infectious diseases. Lancet Infect. Dis. 4, 100–111.

Brzuszkiewicz, E., Thürmer, A., Schuldes, J., Leimbach, A., Liesegang, H., Meyer, F.-D., Boelter, J., Petersen, H., Gottschalk, G., Daniel, R., 2011. Genome sequence analyses of two isolates from the recent Escherichia coli outbreak in Germany reveal the emergence of a new pathotype: Entero-Aggregative-Haemorrhagic Escherichia coli (EAHEC). Arch. Microbiol. 193, 883–91.

Bugarel, M., Vignaud, M.-L., Moury, F., Fach, P., Brisabois, A., 2012. Molecular identification in monophasic and nonmotile variants of *Salmonella enterica* serovar Typhimurium. Microbiologyopen 1, 481–489.

Buitrago, D., Cano-Gomez, C., Aguero, M., Fernandez-Pacheco, P., Gomez-Tejedor, C., Jimenez-Clavero, M. a., Cano-Gómez, C., Agüero, M., Fernandez-Pacheco, P., Gómez-Tejedor, C., Jiménez-Clavero, M.A., Cano-Gomez, C., Aguero, M., Fernandez-Pacheco, P., Gomez-Tejedor, C., Jimenez-Clavero, M. a., 2010. A Survey of Porcine Picornaviruses and Adenoviruses in Fecal Samples in Spain. J. Vet. Diagnostic Investig. 22, 763–766.

Bukholm, G., 1988. Human rotavirus infection enhances invasiveness of enterobacteria in MA-104 cells. APMIS 96, 1118–24.

Burrough, E., Terhorst, S., Sahin, O., Zhang, Q., 2013. Prevalence of Campylobacter spp. relative to other enteric pathogens in grow-finish pigs with diarrhea. Anaerobe 22, 111–114.

Burrough, E.R., Strait, E.L., Kinyon, J.M., Bower, L.P., Madson, D.M., Wilberts, B.L.,

Schwartz, K.J., Frana, T.S., Songer, J.G., 2012. Comparative virulence of clinical Brachyspira spp. isolates in inoculated pigs. J. Vet. Diagn. Invest. 24, 1025–34.

Butler, J.M., Ruitberg, C.M., Vallone, P.M., 2001. Capillary electrophoresis as a tool for optimization of multiplex PCR reactions. Fresenius. J. Anal. Chem. 369, 200–5.

Cacciò, S.M., De Giacomo, M., Pozio, E., 2002. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction–restriction fragment length polymorphism assay to genotype Giardia duodenalis cysts from human faecal samples. Int. J. Parasitol. 32, 1023–1030.

Cadar, D., Cságola, A., Kiss, T., Tuboly, T., 2013. Capsid protein evolution and comparative phylogeny of novel porcine parvoviruses. Mol. Phylogenet. Evol. 66, 243–53.

Cadar, D., Cságola, A., Lorincz, M., Tombácz, K., Kiss, T., Spînu, M., Tuboly, T., 2011. Genetic detection and analysis of porcine bocavirus type 1 (PoBoV1) in European wild boar (Sus scrofa). Virus Genes 43, 376–9.

Cady, N.C., Stelick, S., Kunnavakkam, M. V., Batt, C.A., 2005. Real-time PCR detection of Listeria monocytogenes using an integrated microfluidics platform. Sensors Actuators B Chem. 107, 332–341.

Calderaro, A., Bommezzadri, S., Gorrini, C., Piccolo, G., Peruzzi, S., Dettori, G., Chezzi, C., 2006. Comparative evaluation of molecular assays for the identification of intestinal spirochaetes from diseased pigs. Vet. Microbiol. 118, 91–100.

Call, D.R., Borucki, M.K., Loge, F.J., 2003. Detection of bacterial pathogens in environmental samples using DNA microarrays. J. Microbiol. Methods 53, 235–243.

Campagna, M., Eichwald, C., Vascotto, F., Burrone, O.R., 2005. RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle. J. Gen. Virol. 86, 1481–7.

Cano-Gómez, C., Buitrago, D., Fernández-Pinero, J., Fernández-Pacheco, P., Mansilla, C., Agüero, M., Jiménez-Clavero, M.A., 2011a. Evaluation of a fluorogenic real-time reverse transcription-polymerase chain reaction method for the specific detection of all known serotypes of porcine teschoviruses. J. Virol. Methods 176, 131–4.

Cano-Gómez, C., García-Casado, M.A., Soriguer, R., Palero, F., Jiménez-Clavero, M.A., 2013. Teschoviruses and sapeloviruses in faecal samples from wild boar in Spain. Vet. Microbiol. 165, 115–22.

Cano-Gómez, C., Palero, F., Buitrago, M.D., García-Casado, M.A., Fernández-Pinero, J., Fernández-Pacheco, P., Agüero, M., Gómez-Tejedor, C., Jiménez-Clavero, M.Á., 2011b. Analyzing the genetic diversity of teschoviruses in Spanish pig populations

using complete VP1 sequences. Infect. Genet. Evol. 11, 2144–50.

Carman, P.S., Hazlett, M.J., 1992. Bovine coronavirus infection in Ontario 1990-1991. Can. Vet. journal. La Rev. vétérinaire Can. 33, 812–4.

Carman, S., van Dreumel, T., Ridpath, J., Hazlett, M., Alves, D., Dubovi, E., Tremblay, R., Bolin, S., Godkin, A., Anderson, N., 1998. Severe acute bovine viral diarrhea in Ontario, 1993-1995. J Vet Diagn Invest 10, 27–35.

Caron, Y., Delleur, V., Cassart, D., Losson, B., Laitat, M., 2014. A case of trichurosis in gilts and fattening pigs. JMM Case Reports 1.

Carstens, E.B., 2010. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). Arch. Virol. 155, 133–46.

Carter, G.P., Chakravorty, A., Pham Nguyen, T.A., Mileto, S., Schreiber, F., Li, L., Howarth, P., Clare, S., Cunningham, B., Sambol, S.P., Cheknis, A., Figueroa, I., Johnson, S., Gerding, D., Rood, J.I., Dougan, G., Lawley, T.D., Lyras, D., 2015. Defining the Roles of TcdA and TcdB in Localized Gastrointestinal Disease, Systemic Organ Damage, and the Host Response during Clostridium difficile Infections. MBio 6, e00551.

Cavanagh D., 2005. Coronaviridae: a review of coronaviruses and toroviruses. In: Schmidt, A., Wolff, M.H., Weber, O.F., Weber, O. (Eds.), Coronaviruses with Special Emphasis on First Insights Concerning SARS. Birkhäuser Verlag, p. 232.

Chae, C., Kim, O., Choi, C., Min, K., Cho, W.S., Kim, J., Tai, J.H., 2000. Prevalence of porcine epidemic diarrhoea virus and transmissible gastroenteritis virus infection in Korean pigs. Vet. Rec. 147, 606–8.

Chae, J.-S., Choi, K.-S., 2010. Genetic diversity of porcine circovirus type 2 from pigs in Republic of Korea. Res. Vet. Sci. 88, 333–8.

Chan, G., Farzan, A., Prescott, J.F., Friendship, R., 2013. Brief Communication Communication brève 54.

Chan, G., Farzan, A., Soltes, G., Nicholson, V.M., Pei, Y., Friendship, R., Prescott, J.F., 2012. The epidemiology of Clostridium perfringens type A on Ontario swine farms, with special reference to cpb2-positive isolates. BMC Vet. Res. 8, 156.

Chan, W.C., Nie, S., 1998. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 281, 2016–8.

Chandler-Bostock, R., Hancox, L.R., Nawaz, S., Watts, O., Iturriza-Gomara, M., Mellits, K.M., 2014. Genetic diversity of porcine group A rotavirus strains in the UK. Vet. Microbiol. 173, 27–37.

Chard, L.S., Bordeleau, M.E., Pelletier, J., Tanaka, J., Belsham, G.J., 2006. Hepatitis C virus-related internal ribosome entry sites are found in multiple genera of the

family Picornaviridae. J. Gen. Virol. 87, 927–936.

Chen, C., Grennan, K., Badner, J., Zhang, D., Gershon, E., Jin, L., Liu, C., 2011. Removing batch effects in analysis of expression microarray data: An evaluation of six batch adjustment methods. PLoS One 6.

Chen, E.C., Yagi, S., Kelly, K.R., Mendoza, S.P., Maninger, N., Rosenthal, A., Spinner, A., Bales, K.L., Schnurr, D.P., Lerche, N.W., Chiu, C.Y., 2011. Cross-Species Transmission of a Novel Adenovirus Associated with a Fulminant Pneumonia Outbreak in a New World Monkey Colony. PLoS Pathog. 7, e1002155.

Chen, H., 2009. Diagnosis and treatment of porcine transmissible gastroenteritis. Anim. Husb. Feed Sci. 1, 22–28.

Chen, J., Chen, F., Zhou, Q., Li, W., Song, Y., Pan, Y., Zhang, X., Xue, C., Bi, Y., Cao, Y., 2012. Complete Genome Sequence of a Novel Porcine Sapelovirus Strain YC2011 Isolated from Piglets with Diarrhea. J. Virol. 86, 10898–10898.

Chen, J., Chen, F., Zhou, Q., Li, W., Song, Y., Pan, Y., Zhang, X., Xue, C., Bi, Y., Cao, Y., 2012. Complete Genome Sequence of a Novel Porcine Sapelovirus Strain YC2011 Isolated from Piglets with Diarrhea. J. Virol. 86, 10898–10898.

Chen, Q., Li, J., Fang, X.-E., Xiong, W., 2010. Detection of swine transmissible gastroenteritis coronavirus using loop-mediated isothermal amplification. Virol. J. 7, 206.

Chen, S., Lo, C., Su, S., Kuo, B., Hsiung, C., Lin, C., 2010. UPS 2.0: unique probe selector for probe design and oligonucleotide microarrays at the pangenomic/genomic level. BMC Genomics 11 Suppl 4, S6.

Chen, S., Zhao, S., McDermott, P.F., Schroeder, C.M., White, D.G., Meng, J., 2005. A DNA microarray for identification of virulence and antimicrobial resistance genes in Salmonella serovars and Escherichia coli. Mol. Cell. Probes 19, 195–201.

Chen, T.-C., Chen, G.-W., Hsiung, C.A., Yang, J.-Y., Shih, S.-R., Lai, Y.-K., Juang, J.-L., 2006. Combining multiplex reverse transcription-PCR and a diagnostic microarray to detect and differentiate enterovirus 71 and coxsackievirus A16. J. Clin. Microbiol. 44, 2212–9.

Cheng, W.X., Li, J.S., Huang, C.P., Yao, D.P., Liu, N., Cui, S.X., Jin, Y., Duan, Z.J., 2010. Identification and nearly full-length genome characterization of novel porcine bocaviruses. PLoS One 5, 1–8.

Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch. Virol. 152, 1035–44.

Chiu, C.Y., Greninger, A.L., Kanada, K., Kwok, T., Fischer, K.F., Runckel, C., Louie, J.K.,

Glaser, C.A., Yagi, S., Schnurr, D.P., Haggerty, T.D., Parsonnet, J., Ganem, D., DeRisi, J.L., 2008. Identification of cardioviruses related to Theiler's murine encephalomyelitis virus in human infections. Proc. Natl. Acad. Sci. 105, 14124–14129.

Chiu, S.-C., Hu, S.-C., Chang, C.-Y.C.-C., Chang, C.-Y.C.-C., Huang, C.-C., Pang, V.F., Wang, F.-I., 2012. The role of porcine teschovirus in causing diseases in endemically infected pigs. Vet. Microbiol. 161, 88–95.

Chiu, T.-H., Pang, J.-C., Hwang, W.-Z., Tsen, H.-Y., 2005. Development of PCR primers for the detection of Salmonella enterica serovar Choleraesuis based on the fliC gene. J. Food Prot. 68, 1575–80.

Chou, H.-H., 2010. Shared probe design and existing microarray reanalysis using PICKY. BMC Bioinformatics 11, 196.

Cho, Y.-I.Y.-I.I., Kim, W.-I.W.-I.I., Liu, S., Kinyon, J.M., Yoon, K.J., 2010. Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. J Vet Diagn Invest 22, 509–517.

Cho, Y.-I., Han, J.-I., Wang, C., Cooper, V., Schwartz, K., Engelken, T., Yoon, K.-J., 2013. Case-control study of microbiological etiology associated with calf diarrhea. Vet. Microbiol. 166, 375–85.

Choi, M.G., Park, S.J., Nguyen, V.G., Chung, H.C., Kim, A.R., Park, B.K., 2014. Molecular detection and genetic analysis of porcine bocavirus in Korean domestic swine herds. Arch. Virol. 159, 1487–1492.

Chou, H.-H., Hsia, A.-P., Mooney, D.L., Schnable, P.S., 2004. Picky: oligo microarray design for large genomes. Bioinformatics 20, 2893–902.

Clark, M., 1993. Bovine coronavirus. Br. Vet. J. 149, 51–70.

Collins, J.E., Bergeland, M.E., Myers, L.L., Shoop, D.S., 1989. Exfoliating Colitis Associated with Enterotoxigenic Bacteroides Fragilis in a Piglet. J. Vet. Diagnostic Investig. 1, 349–351.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H., Farrow, J.A., 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int J Syst. Bacteriol 44, 812–826.

Collins, A.M.; Love, R.J. Re-challenge of pigs following recovery from proliferative enteropathy. Vet. Microbiol. 120, 381–386.

Coppée, J.-Y., 2008. Do DNA microarrays have their future behind them? Microbes Infect. 10, 1067–1071.

Cotmore, S.F., Agbandje-McKenna, M., Chiorini, J.A., Mukha, D. V., Pintel, D.J., Qiu, J., Soderlund-Venermo, M., Tattersall, P., Tijssen, P., Gatherer, D., Davison, A.J., 2014. The family Parvoviridae. Arch. Virol. 159, 1239–1247.

Coulson, B.S., 1996. VP4 and VP7 typing using monoclonal antibodies. Arch. Virol. Suppl. 12, 113–8.

Coursodon, C.F., Glock, R.D., Moore, K.L., Cooper, K.K., Songer, J.G., 2012. TpeL-producing strains of Clostridium perfringens type A are highly virulent for broiler chicks. Anaerobe 18, 117–121.

Crouch, C.F., 1985. Vaccination against enteric rota and coronaviruses in cattle and pigs: Enhancement of lactogenic immunity. Vaccine 3, 284–291.

Crouch, C.F., Acres, S.D., 1984. Prevalence of rotavirus and coronavirus antigens in the feces of normal cows. Can. J. Comp. Med. Rev. Can. médecine Comp. 48, 340–2.

Crouch, C.F., Woode, G.N., 1978. Serial studies of virus multiplication and intestinal damage in gnotobiotic piglets infected with rotavirus. J. Med. Microbiol. 11, 325–34.

Cruz Junior, E.C., Salvarani, F.M., Silva, R.O.S., Silva, M.X., Lobato, F.C.F., Guedes, R.M.C., 2013. A surveillance of enteropathogens in piglets from birth to seven days of age in Brazil. Pesqui. Veterinária Bras. 33, 963–969.

Cunningham, S.A., Sloan, L.M., Nyre, L.M., Vetter, E.A., Mandrekar, J., Patel, R., 2010. Three-hour molecular detection of Campylobacter, Salmonella, Yersinia, and Shigella species in feces with accuracy as high as that of culture. J. Clin. Microbiol. 48, 2929–33.

Cutillas, C., de Rojas, M., Ariza, C., Ubeda, J.M., Guevara, D., 2007. Molecular identification of Trichuris vulpis and Trichuris suis isolated from different hosts. Parasitol. Res. 100, 383–9.

Dabisch-Ruthe, M., Vollmer, T., Adams, O., Knabbe, C., Dreier, J., 2012. Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG respiratory virus panel fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay. BMC Infect. Dis. 12, 163.

Dai, Y., Zhou, Q., Zhang, C., Song, Y., Tian, X., Zhang, X., Xue, C., Xu, S., Bi, Y., Cao, Y., 2012. Complete genome sequence of a porcine orthoreovirus from southern China. J. Virol. 86, 12456.

Dalma-Weiszhausz, D.D., Warrington, J., Tanimoto, E.Y., Miyada, C.G., 2006. [1] The Affymetrix GeneChip® Platform: An Overview. Methods Enzymol. 410, 3–28.

Danbara, H., Moriguchi, R., Suzuki, S., Tamura, Y., Kijima, M., Oishi, K., Matsui, H., Abe, A., Nakamura, M., 1992. Effect of 50 kilobase-plasmid, pKDSC50, of Salmonella

choleraesuis RF-1 strain on pig septicemia. J. Vet. Med. Sci. 54, 1175–8.

Danckert, L., Hoppe, S., Bier, F.F., von Nickisch-Rosenegk, M., 2014. Rapid identification of novel antigens of Salmonella Enteritidis by microarray-based immunoscreening. Mikrochim. Acta 181, 1707–1714.

Daniel, R., Matthews, L., Willshaw, G., 1998. Isolation of E coli O157 from a calf with dysentery. Vet. Rec. 143, 56.

Darkoh, C., Dupont, H.L., Kaplan, H.B. 2011. Novel One-Step Method for Detection and Isolation of Active-Toxin-Producing Clostridium difficile Strains Directly from Stool Samples. J Clin Microbiol. 49(12), 4219–4224

Darweesh, M.F., Rajput, M.K.S., Braun, L.J., Ridpath, J.F., Neill, J.D., Chase, C.C.L., 2015. Characterization of the cytopathic BVDV strains isolated from 13 mucosal disease cases arising in a cattle herd. Virus Res. 195, 141–147.

Das, A., Spackman, E., Pantin-Jackwood, M.J., Suarez, D.L., 2009. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR. J. Vet. Diagn. Invest. 21, 771–8.

Das, B.K., Gentsch, J.R., Cicirello, H.G., Woods, P.A., Gupta, A., Ramachandran, M., Kumar, R., Bhan, M.K., Glass2, R.I., 1994. Characterization of Rotavirus Strains from Newborns in New Delhi, India. J. Clin. Microbiol. 32, 1820–1822.

Daugschies, A., Najdrowski, M., 2005. Eimeriosis in cattle: current understanding. J. Vet. Med. B. Infect. Dis. Vet. Public Health 52, 417–27.

de la Fuente, R., García, A., Ruiz-Santa-Quiteria, J.., Luzón, M., Cid, D., García, S., Orden, J.., Gómez-Bautista, M., 1998. Proportional morbidity rates of enteropathogens among diarrheic dairy calves in central Spain. Prev. Vet. Med. 36, 145–152.

de la Fuente, R., Luzón, M., Ruiz-Santa-Quiteria, J.., García, A., Cid, D., Orden, J.., García, S., Sanz, R., Gómez-Bautista, M., 1999. Cryptosporidium and concurrent infections with other major enterophatogens in 1 to 30-day-old diarrheic dairy calves in central Spain. Vet. Parasitol. 80, 179–185.

Debast, S.B., Van Leengoed, L.A.M.G., Goorhuis, A., Harmanus, C., Kuijper, E.J., Bergwerff, A.A., 2009. Clostridium difficile PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. Environ. Microbiol. 11, 505–511.

Debaugnies, F., Busson, L., Ferster, A., Lewalle, P., Azzi, N., Aoun, M., Verhaegen, G., Mahadeb, B., De Marchin, J., Vandenberg, O., Hallin, M., 2014. Detection of Herpesviridae in whole blood by multiplex PCR DNA-based microarray analysis after hematopoietic stem cell transplantation. J. Clin. Microbiol. 52, 2552–2556.

Decaro, N., Campolo, M., Desario, C., Cirone, F., D'Abramo, M., Lorusso, E., Greco, G., Mari, V., Colaianni, M.L., Elia, G., Martella, V., Buonavoglia, C., 2008a. Respiratory disease associated with bovine coronavirus infection in cattle herds in Southern Italy. J. Vet. Diagn. Invest. 20, 28–32.

Decaro, N., Campolo, M., Desario, C., Ricci, D., Camero, M., Lorusso, E., Elia, G., Lavazza, A., Martella, V., Buonavoglia, C., 2005. Virological and molecular characterization of a mammalian orthoreovirus type 3 strain isolated from a dog in Italy. Vet. Microbiol. 109, 19–27.

Decaro, N., Elia, G., Campolo, M., Desario, C., Mari, V., Radogna, A., Colaianni, M.L., Cirone, F., Tempesta, M., Buonavoglia, C., 2008b. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J. Virol. Methods 151, 167–71.

Decaro, N., Mari, V., Desario, C., Campolo, M., Elia, G., Martella, V., Greco, G., Cirone, F., Colaianni, M.L., Cordioli, P., Buonavoglia, C., 2008c. Severe outbreak of bovine coronavirus infection in dairy cattle during the warmer season. Vet. Microbiol. 126, 30–39.

Deng, Y., He, Z., Van Nostrand, J.D., Zhou, J., 2008. Design and analysis of mismatch probes for long oligonucleotide microarrays. BMC Genomics 9, 491.

Denis, M., Henrique, E., Chidaine, B., Tircot, A., Bougeard, S., Fravalo, P., 2011. Campylobacter from sows in farrow-to-finish pig farms: Risk indicators and genetic diversity. Vet. Microbiol. 154, 163–170.

Denis, M., Soumet, C., Rivoal, K., Ermel, G., Blivet, D., Salvat, G., Colin, P., 1999. Development of a m-PCR assay for simultaneous identification of Campylobacter jejuni and C. coli. Lett. Appl. Microbiol. 29, 406–410.

Derveaux, S., Vandesompele, J., Hellemans, J., 2010. How to do successful gene expression analysis using real-time PCR. Methods 50, 227–230.

Dewey, C.E., Johnston, W.T., Gould, L., Whiting, T.L., 2006. Postweaning mortality in Manitoba swine. Can. J. Vet. Res. = Rev. Can. Rech. vétérinaire 70, 161–7.

Di Profio, F., Ceci, C., Di Felice, E., Marsilio, F., Di Martino, B., 2013. Molecular detection of porcine kobuviruses in Italian swine. Res. Vet. Sci. 95, 782–785.

Diaz-Sanchez, S., Hanning, I., Pendleton, S., D'Souza, D., 2013. Next-generation sequencing: the future of molecular genetics in poultry production and food safety. Poult. Sci. 92, 562–72.

Diéguez, F.J., Yus, E., Vilar, M.J., Sanjuán, M.L., Arnaiz, I., 2009. Effect of the bovine viral diarrhoea virus (BVDV) infection on dairy calf rearing. Res. Vet. Sci. 87, 39–40.

Dilovski, M., Ognianov, D., 1975. [Isolation and identification of a virus causing abortions and stillbirths in swine (Preliminary report)]. Vet. Med. Nauki 12, 52–3.

Dimitrov, R. a, Zuker, M., 2004. Prediction of hybridization and melting for double-stranded nucleic acids. Biophys. J. 87, 215–26.

Dittmar, M., Hoelzle, L.E., Hoelzle, K., Sydler, T., Corboz, L., Miserez, R., Wittenbrink, M.M., 2003. Diagnosis of Porcine Proliferative Enteropathy: Detection of Lawsonia intracellularis by Pathological Examinations, Polymerase Chain Reaction and Cell Culture Inoculation. J. Vet. Med. Ser. B 50, 332–338.

Donatin, E., Buffet, S., Leroy, Q., Raoult, D., Drancourt, M., 2013. A DNA microarray for the versatile diagnosis of infectious diarrhea. APMIS 121, 634–42.

Donnenberg, M.S., Kaper, J.B., 1992. Enteropathogenic Escherichia coli. Infect.Immun 60, 3953–3961.

Dorris, D.R., Nguyen, A., Gieser, L., Lockner, R., Lublinsky, A., Patterson, M., Touma, E., Sendera, T.J., Elghanian, R., Mazumder, A., 2003. Oligodeoxyribonucleotide probe accessibility on a three-dimensional DNA microarray surface and the effect of hybridization time on the accuracy of expression ratios. BMC Biotechnol. 3, 6.

DOYLE, L.P., HUTCHINGS, L.M., 1946. A transmissible gastroenteritis in pigs. J. Am. Vet. Med. Assoc. 108, 257–9.

Drobyshev, A.L., Machka, C., Horsch, M., Seltmann, M., Liebscher, V., Hrabé de Angelis, M., Beckers, J., 2003. Specificity assessment from fractionation experiments (SAFE): a novel method to evaluate microarray probe specificity based on hybridisation stringencies. Nucleic Acids Res. 31, E1–E1.

Drosten, C., Günther, S., Preiser, W., van der Werf, S., Brodt, H.-R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R.A.M., Berger, A., Burguière, A.-M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J.-C., Müller, S., Rickerts, V., Stürmer, M., Vieth, S., Klenk, H.-D., Osterhaus, A.D.M.E., Schmitz, H., Doerr, H.W., 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. 348, 1967–76.

Duarte, M., Tobler, K., Bridgen, A., Rasschaert, D., Ackermann, M., Laude, H., 1994. Sequence Analysis of the Porcine Epidemic Diarrhea Virus Genome between the Nucleocapsid and Spike Protein Genes Reveals a Polymorphic ORF. Virology 198, 466–476.

Dubberke, E.R., Haslam, D.B., Lanzas, C., Bobo, L.D., Burnham, C.-A.D., Gröhn, Y.T., Tarr, P.I., 2011. The ecology and pathobiology of Clostridium difficile infections: an interdisciplinary challenge. Zoonoses Public Health 58, 4–20.

Dubinsky, E.A., Esmaili, L., Hulls, J.R., Cao, Y., Griffith, J.F., Andersen, G.L., 2012.

Application of phylogenetic microarray analysis to discriminate sources of fecal pollution. Environ. Sci. Technol. 46, 4340–7.

Dufkova, L., Scigalkova, I., Moutelikova, R., Malenovska, H., Prodelalova, J., 2013. Genetic diversity of porcine sapoviruses, kobuviruses, and astroviruses in asymptomatic pigs: an emerging new sapovirus GIII genotype. Arch. Virol. 158, 549–58.

Dufva, M., Petersen, J., Poulsen, L., 2009. Increasing the specificity and function of DNA microarrays by processing arrays at different stringencies. Anal. Bioanal. Chem. 395, 669–77.

Duimstra, J.R., Myers, L.L., Collins, J.E., Benfield, D. a., Shoop, D.S., Bradbury, W.C., 1991. Enterovirulence of Enterotoxigenic Bacteroides fragilis in Gnotobiotic Pigs. Vet. Pathol. 28, 514–518.

Duncan, R., Corcoran, J., Shou, J., Stoltz, D., 2004. Reptilian reovirus: A new fusogenic orthoreovirus species. Virology 319, 131–140.

Dunne, H.W., Ajinkya, S.M., Bubash, G.R., Griel, L.C., 1973. Parainfluenza 3 and bovine enteroviruses as possible important causative factors in bovine abortion. Am. J. Vet. Res. 34, 1121–1126.

Dunne, H.W., Huang, C.M., Lin, W.J., 1974. Bovine enteroviruses in the calf: an attempt at serologic, biologic, and pathologic classification. J. Am. Vet. Med. Assoc. 164, 290–4.

Dupont, K., Nielsen, E.O., Bækbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case–control study supports a shift in genotypes with time. Vet. Microbiol. 128, 56–64.

Durham, P.J., Hassard, L.E., Norman, G.R., Yemen, R.L., 1989. Viruses and virus-like particles detected during examination of feces from calves and piglets with diarrhea. Can. Vet. journal. La Rev. vétérinaire Can. 30, 876–81.

Durham, P.J.K., Johnson, R.H., Parker, R.J., 1985a. Exacerbation of experimental parvoviral enteritis in calves by coccidia and weaning stress. Res. Vet. Sci. 39, 16–23.

Durham, P.J.K., Lax, A., Johnson, R.H., 1985b. Pathological and virological studies of experimental parvoviral enteritis in calves. Res. Vet. Sci. 38, 209–219.

Ehrlich, S.D. 2016. The human gut microbiome impacts health and disease. C R Biol. Jul-Aug;339 (7-8):319-23. doi: 10.1016/j.crvi.2016.04.008. Epub 2016 May 25.

Siddell S., and Snijder E.J. 2008. An introduction to Nidoviruses. In: Perlman S., G.T. and S.E.. (Ed.), Nidoviruses. ASM Press, Washington DC, pp. 1–13.

Eijck, I.A.J.M., Borgsteede, F.H.M., 2005. A survey of gastrointestinal pig parasites on

free-range, organic and conventional pig farms in The Netherlands. Vet. Res. Commun. 29, 407–14.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. journal. La Rev. vétérinaire Can. 39, 44–51.

Ellsworth, D.L.; Rittenhouse, K.D., Honeycutt, R.L., 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques, 14: 214-217.

Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E., 2000. Multiplex PCR: optimization and application in diagnostic virology. Clin. Microbiol. Rev. 13, 559–70.

El-Seedy, F.R., Abed, A.H., Yanni, H.A., Abd El-Rahman, S.A.A., 2016. Prevalence of Salmonella and E. coli in neonatal diarrheic calves. Beni-Suef Univ. J. Basic Appl. Sci. 5, 45–51.

Enemark, H.L., Dahl, J., Enemark, J.M.D., 2013. Eimeriosis in Danish Dairy Calves – Correlation between Species, Oocyst Excretion and Diarrhoea. Parasitol. Res. 112, 169–176.

Erwanto, Y., Abidin, M.Z., Sismindari, X., Rohman, A., 2012. Pig species identification in meatballs using polymerase chain reaction- restriction fragment length polymorphism for Halal authentication. Int. Food Res. J. 19, 901–906.

Estes, M., Kapikian, A., 2007. Rotaviruses. In: Knipe, D., Griffin, D., Lamb, R., Martin, M., Roizman, B., Straus, S. (Eds.), Fields Virology. Wolters Kluwer Health; Lippincott, Williams and Wilkins, Philadelphia, pp. 1917–1975.

Eswarappa, S.M., Janice, J., Nagarajan, A.G., Balasundaram, S. V., Karnam, G., Dixit, N.M., Chakravortty, D., 2008. Differentially evolved genes of Salmonella pathogenicity islands: Insights into the mechanism of host specificity in Salmonella. PLoS One 3.

Euzéby, J.P., 2013. List of Prokaryotic names with Standing in Nomenclature [WWW Document]. URL Available online at: www.bacterio.net (accessed 5.1.16).

Fan, J., Gunderson, K.L., Bibikova, M., Yeakley, J.M., Chen, J., Wickham Garcia, E., Lebruska, L.L., Laurent, M., Shen, R., Barker, D., 2006a. Illumina Universal Bead Arrays. Methods Enzymol. 410, 57–73.

Fan, J.-B., Chee, M.S., Gunderson, K.L., 2006b. Highly parallel genomic assays. Nat. Rev. Genet. 7, 632–44.

Farkas, T., Nakajima, S., Sugieda, M., Deng, X., Zhong, W., Jiang, X., 2005.

Seroprevalence of noroviruses in swine. J. Clin. Microbiol. 43, 657–61.

Farkas, T., Zhong, W.M., Jing, Y., Huang, P.W., Espinosa, S.M., Martinez, N., Morrow, A.L., Ruiz-Palacios, G.M., Pickering, L.K., Jiang, X., 2004. Genetic diversity among sapoviruses. Arch. Virol. 149, 1309–1323.

Farzan, A., Kircanski, J., DeLay, J., Soltes, G., Songer, J.G., Friendship, R., Prescott, J.F., 2013. An investigation into the association between cpb2-encoding Clostridium perfringens type A and diarrhea in neonatal piglets. Can. J. Vet. Res. 77, 45–53.

Fauquet, C.M., Mayo, M. a, Maniloff, J., Desselberger, U., Ball, L. a, 2005. Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Virus Res. 83, 221–222.

Fayer, R., 2010. Taxonomy and species delimitation in Cryptosporidium. Exp. Parasitol. 124, 90–97.

Feehery, G.R., Yigit, E., Oyola, S.O., Langhorst, B.W., Schmidt, V.T., Stewart, F.J., Dimalanta, E.T., Amaral-Zettler, L.A., Davis, T., Quail, M.A., Pradhan, S., 2013. A Method for Selectively Enriching Microbial DNA from Contaminating Vertebrate Host DNA. PLoS One 8.

Fellström, C., Gunnarsson, A., 1995. Phenotypical characterisation of intestinal spirochaetes isolated from pigs. Res. Vet. Sci. 59, 1–4.

Fenart, S., Chabi, M., Gallina, S., Huis, R., Neutelings, G., Riviere, N., Thomasset, B., Hawkins, S., Lucau-Danila, A., 2013. Intra-platform comparison of 25-mer and 60-mer oligonucleotide Nimblegen DNA microarrays. BMC Res. Notes 6, 43.

Feng, Y., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of Giardia species and giardiasis. Clin. Microbiol. Rev. 24, 110–40.

Fenner, FJ, Gibbs, GJ, Murphy, FA, Rott, R, Studdert, MJ, White, D., 1993. Porcine enteroviruses. In: Al., F. et (Ed.), Veterinary Virology. Academic Press, San Diego, pp. 416–419.

Fernandes, L.G., Nogueira, A.H. de C., De Stefano, E., Pituco, E.M., Ribeiro, C.P., Alves, C.J., Oliveira, T.S., Clementino, I.J., de Azevedo, S.S., 2016. Herd-level prevalence and risk factors for bovine viral diarrhea virus infection in cattle in the State of Paraíba, Northeastern Brazil. Trop. Anim. Health Prod. 48, 157–65.

Finlaison, D., Kirkland, P., Luong, R., Ross, A., 2007. Survey of porcine circovirus 2 and postweaning multisystemic wasting syndrome in New South Wales piggeries. Aust. Vet. J. 85, 304–10.

Fish, D.J., Horne, M.T., Brewood, G.P., Goodarzi, J.P., Alemayehu, S., Bhandiwad, A., Searles, R.P., Benight, A.S., 2007. DNA multiplex hybridization on microarrays and thermodynamic stability in solution: a direct comparison. Nucleic Acids Res. 35,

7197–208.

Flanegan, J.B., Petterson, R.F., Ambros, V., Hewlett, N.J., Baltimore, D., 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5'-terminus of virion and replicative intermediate RNAs of poliovirus. Proc. Natl. Acad. Sci. U. S. A. 74, 961–5.

Flynn, W.T., Saif, L.J., Moorhead, P.D., 1988. Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs. Am. J. Vet. Res. 49, 819–25.

Fodor, S.P., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T., Solas, D., 1991. Light-directed, spatially addressable parallel chemical synthesis. Science 251, 767–73.

Fookes, M., Schroeder, G.N., Langridge, G.C., Blondel, C.J., Mammina, C., Connor, T.R., Seth-Smith, H., Vernikos, G.S., Robinson, K.S., Sanders, M., Petty, N.K., Kingsley, R.A., B??umler, A.J., Nuccio, S.P., Contreras, I., Santiviago, C.A., Maskell, D., Barrow, P., Humphrey, T., Nastasi, A., Roberts, M., Frankel, G., Parkhill, J., Dougan, G., Thomson, N.R., 2011. Salmonella bongori provides insights into the evolution of the salmonellae. PLoS Pathog. 7.

Forman, A.J., Pass, D.A., Connaughton, I.D., 1982. The characterisation and pathogenicity of porcine enteroviruses isolated in Victoria. Aust. Vet. J. 58, 136–42.

Forsdyke, D.R., 2007. Calculation of folding energies of single-stranded nucleic acid sequences: Conceptual issues. J. Theor. Biol. 248, 745–753.

Fossler, C.P., Wells, S.J., Kaneene, J.B., Ruegg, P.L., Warnick, L.D., Bender, J.B., Eberly, L.E., Godden, S.M., Halbert, L.W., 2005. Herd-level factors associated with isolation of Salmonella in a multi-state study of conventional and organic dairy farms: II. Salmonella shedding in calves. Prev. Vet. Med. 70, 279–291.

Foster, D.M., Smith, G.W., 2009. Pathophysiology of Diarrhea in Calves. Vet. Clin. North Am. Food Anim. Pract. 25, 13–36.

Fotin, a. V., Drobyshev, a. L., Proudnikov, D.Y., Perov, a. N., Mirzabekov, a. D., 1998. Parallel thermodynamic analysis of duplexes on oligodeoxyribonucleotide microchips. Nucleic Acids Res. 26, 1515–1521.

Fournier, P.-E., Dubourg, G., Raoult, D., 2014. Clinical detection and characterization of bacterial pathogens in the genomics era. Genome Med. 6, 114.

Franco, A.A., Cheng, R.K., Chung, G.T., Wu, S., Oh, H.B., Sears, C.L., 1999. Molecular evolution of the pathogenicity island of enterotoxigenic Bacteroides fragilis strains. J. Bacteriol. 181, 6623–33.

Francois, P., Bento, M., Vaudaux, P., Schrenzel, J., 2003. Comparison of fluorescence and resonance light scattering for highly sensitive microarray detection of

bacterial pathogens. J. Microbiol. Methods 55, 755–762.

Fukai, K., Sakai, T., Hirose, M., Itou, T., 1999. Prevalence of calf diarrhea caused by bovine group A rotavirus carrying G serotype 8 specificity. Vet. Microbiol. 66, 301–311.

Fukushima, H., Nakamura, R., Ito, Y., Saito, K., Tsubokura, M., Otsuki, K., 1984. Ecological studies of Yersinia enterocolitica. II. Experimental infection with Y. enterocolitica in pigs. Vet. Microbiol. 9, 375–381.

Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.-H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Can. Vet. journal. La Rev. vétérinaire Can. 48, 811–9.

Galav, V., Mishra, N., Dubey, R., Rajukumar, K., Pitale, S.S., Shrivastav, A.B., Pradhan, H.K., 2007. Pathogenicity of an Indian isolate of bovine viral diarrhea virus 1b in experimentally infected calves. Res. Vet. Sci. 83, 364–368.

Gannon, V.P., Rashed, M., King, R.K., Thomas, E.J., 1993. Detection and characterization of the eae gene of Shiga-like toxin-producing Escherichia coli using polymerase chain reaction. J. Clin. Microbiol. 31, 1268–1274.

García, a., Ruiz-Santa-Quiteria, J. a., Orden, J. a., Cid, D., Sanz, R., Gómez-Bautista, M., De La Fuente, R., 2000. Rotavirus and concurrent infections with other enteropathogens in neonatal diarrheic dairy calves in Spain. Comp. Immunol. Microbiol. Infect. Dis. 23, 175–183.

García-Sánchez, J., Corral, C., Halaihel, N.G., Simon, M.C., Alonso, J.L., Muzquiz, J.L., Ortega, C., Girones, O., 1993. Survey of rotavirus infection in a dairy herd: comparison between polyacrylamide gel electrophoresis and two commercial tests. Vet. Microbiol. 34, 321–32.

Garhyan, J., Gharaibeh, R.Z., McGee, S., Gibas, C.J., 2013. The illusion of specific capture: surface and solution studies of suboptimal oligonucleotide hybridization. BMC Res. Notes 6, 72.

Gava, D., Souza, C.K., Schaefer, R., Vincent, A.L., Cantão, M.E., Coldebella, A., Ciacci-Zanella, J.R., 2015. A TaqMan-based real-time PCR for detection and quantification of porcine parvovirus 4. J. Virol. Methods 219, 14–17.

Gélinas, A.-M., Boutin, M., Sasseville, A.M.-J., Dea, S., 2001. Bovine coronaviruses associated with enteric and respiratory diseases in Canadian dairy cattle display different reactivities to anti-HE monoclonal antibodies and distinct amino acid changes in their HE, S and ns4.9 protein. Virus Res. 76, 43–57.

Gentsch, J.R., Glass, R.I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B.K., Bhan, M.K., 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J. Clin. Microbiol. 30, 1365–73.

Gentsch, J.R., Laird, A.R., Bielfelt, B., Griffin, D.D., Banyai, K., Ramachandran, M., Jain, V., Cunliffe, N. a, Nakagomi, O., Kirkwood, C.D., Fischer, T.K., Parashar, U.D., Bresee, J.S., Jiang, B., Glass, R.I., 2005. Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. J. Infect. Dis. 192 Suppl , S146–S159.

Geyer, A., Sebata, T., Peenze, I., Steele, A.D., 1996. Group B and C porcine rotaviruses identified for the first time in South Africa. J. S. Afr. Vet. Assoc. 67, 115–6.

Gharaibeh, R.Z., Newton, J.M., Weller, J.W., Gibas, C.J., 2010. Application of equilibrium models of solution hybridization to microarray design and analysis. PLoS One 5, e11048.

Gilbert, J.M., Greenberg, H.B., 1998. Cleavage of rhesus rotavirus VP4 after arginine 247 is essential for rotavirus-like particle-induced fusion from without. J. Virol. 72, 5323–7.

Gillhuber, J., Rügamer, D., Pfister, K., Scheuerle, M.C., 2014. Giardiosis and other enteropathogenic infections: a study on diarrhoeic calves in Southern Germany. BMC Res. Notes 7, 112.

Goens, S.D., Botero, S., Zemla, A., Zhou, C.E., Perdue, M.L., 2004. Bovine enterovirus 2: complete genomic sequence and molecular modelling of a reference strain and a wild-type isolate from endemically infected US cattle. J Gen Virol 85, 3195–3203.

Gómez-Duarte, O.G., Bai, J., Newell, E., 2009. Detection of Escherichia coli, Salmonella spp., Shigella spp., Yersinia enterocolitica, Vibrio cholerae, and Campylobacter spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagn. Microbiol. Infect. Dis. 63, 1–9.

Gonçalves, E.M.N., Araújo, R.S., Orban, M., Matté, G.R., Matté, M.H., Corbett, C.E.P., 2008. Protocol for DNA extraction of Cryptosporidium spp. oocysts in fecal samples. Rev. Inst. Med. Trop. Sao Paulo 50, 165–167.

Gong, P., Levicky, R., 2008. DNA surface hybridization regimes. Proc. Natl. Acad. Sci. U. S. A. 105, 5301–5306.

Gonzalez, I., Grant, K.A., Richardson, P.T., Park, S.F., Collins, M.D., 1997. Specific identification of the enteropathogens Campylobacter jejuni and Campylobacter coli by using a PCR test based on the ceuE gene encoding a putative virulence determinant. J. Clin. Microbiol. 35, 759–63.

Goodfellow, I., 2011. The genome-linked protein VPg of vertebrate viruses - A multifaceted protein. Curr. Opin. Virol. 1, 355–362.

Goosney, D.L., de Grado, M., Finlay, B.B., 1999. Putting E. coli on a pedestal: a unique system to study signal transduction and the actin cytoskeleton. Trends Cell Biol.

9, 11–14.

Gorski, L., Parker, C.T., Liang, A., Cooley, M.B., Jay-Russell, M.T., Gordus, A.G., Atwill, E.R., Mandrell, R.E., 2011. Prevalence, distribution, and diversity of Salmonella enterica in a major produce region of California. Appl. Environ. Microbiol. 77, 2734–48.

Goulding, D., Thompson, H., Emerson, J., Fairweather, N.F., Dougan, G., Douce, G.R., 2009. Distinctive profiles of infection and pathology in hamsters infected with Clostridium difficile strains 630 and B1. Infect. Immun. 77, 5478–85.

Gouvea, V., Allen, J.R., Glass, R.I., Fang, Z.Y., Bremont, M., Cohen, J., McCrae, M.A., Saif, L.J., Sinarachatanant, P., Caul, E.O., 1991. Detection of group B and C rotaviruses by polymerase chain reaction. J. Clin. Microbiol. 29, 519–23.

Gravesen, A., Jacobsen, T., Møller, P.L., Hansen, F., Larsen, A.G., Knøchel, S., 2000. Genotyping of Listeria monocytogenes: Comparison of RAPD, ITS, and PFGE. Int. J. Food Microbiol. 57, 43–51.

Green K.Y., Chanock R.M., K.A.Z., 2001. Fields virology. In: Knipe DM, H.P. (Ed.), Human Caliciviruses. Philadelphia, pp. 841–874.

Greninger, A.L., Chen, E.C., Sittler, T., Scheinerman, A., Roubinian, N., Yu, G., Kim, E., Pillai, D.R., Guyard, C., Mazzulli, T., Isa, P., Arias, C.F., Hackett, J., Schochetman, G., Miller, S., Tang, P., Chiu, C.Y., 2010. A metagenomic analysis of pandemic influenza A (2009 H1N1) infection in patients from North America. PLoS One 5, e13381.

Griessl, M.H., Schmid, B., Kassler, K., Braunsmann, C., Ritter, R., Barlag, B., Stierhof, Y.D., Sturm, K.U., Danzer, C., Wagner, C., Schäffer, T.E., Sticht, H., Hensel, M., Muller, Y.A., 2013. Structural insight into the giant Ca2+-binding adhesin siie: Implications for the adhesion of salmonella enterica to polarized epithelial cells. Structure 21, 741–752.

Gualdi, V., Vezzoli, F., Luini, M., Nisoli, L., 2003. The role of Isospora suis in the ethiology of diarrhoea in suckling piglets. Parasitol. Res. 90 Suppl 3, S163–5.

Guiney, D.G., Fierer, J., 2011. The role of the spv genes in Salmonella pathogenesis. Front. Microbiol. 2.

Guion, C.E., Ochoa, T.J., Walker, C.M., Barletta, F., Cleary, T.G., 2008. Detection of diarrheagenic Escherichia coli by use of melting-curve analysis and real-time multiplex PCR. J. Clin. Microbiol. 46, 1752–7.

Gulig, P.A., Curtiss, R., 1987. Plasmid-associated virulence of Salmonella typhimurium. Infect. Immun. 55, 2891–2901.

Gulliksen, S.M., Lie, K.I., Løken, T., Østerås, O., 2009. Calf mortality in Norwegian dairy

herds. J. Dairy Sci. 92, 2782–2795.

Gumerlock, P.H., Tang, Y.J., Weiss, J.B., Silva, J., 1993. Specific Detection of Toxigenic Strains of Clostridium difficile in Stool Specimens. J. Clin. Microbiol. 31, 507–511.

Gumusova,SO, Yazici,Z, Albayrak,H,Meral, Y., 2007. Rotavirus and coronavirus prevalence in healthy calves and calves with diarrhoea. Med. Wet. 63.

Gunderson, K.L., Kruglyak, S., Graige, M.S., Garcia, F., Kermani, B.G., Zhao, C., Che, D., Dickinson, T., Wickham, E., Bierle, J., Doucet, D., Milewski, M., Yang, R., Siegmund, C., Haas, J., Zhou, L., Oliphant, A., Fan, J.-B., Barnard, S., Chee, M.S., 2004. Decoding randomly ordered DNA arrays. Genome Res. 14, 870–7.

Gunn, L., Collins, P.J., Fanning, S., McKillen, J., Morgan, J., Staines, A., O'Shea, H., 2015. Detection and characterisation of novel bocavirus (genus Bocaparvovirus) and gastroenteritis viruses from asymptomatic pigs in Ireland. Infect. Ecol. Epidemiol. 5, 27270.

Günther, H., Otto, P., Heilmann, P., 1984. [Diarrhea in young calves. 6. Determination of the pathogenicity of a bovine coronavirus and an unidentified icosahedral virus]. Arch. für Exp. Veterinärmedizin 38, 781–92.

Guo, A., Lasaro, M.A., Sirard, J.C., Kraehenbühl, J.P., Schifferli, D.M., 2007. Adhesin-dependent binding and uptake of Salmonella enterica serovar Typhimurium by dendritic cells. Microbiology 153, 1059–1069.

Guo, M., Evermann, J.F., Saif, L.J., 2001a. Detection and molecular characterization of cultivable caliciviruses from clinically normal mink and enteric caliciviruses associated with diarrhea in mink. Arch. Virol. 146, 479–93.

Guo, M., Hayes, J., Cho, K.O., Parwani, a V, Lucas, L.M., Saif, L.J., 2001b. Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. J. Virol. 75, 9239–51.

Ha, Y., Jung, K., Kim, J., Choi, C., Chae, C., 2005. Outbreak of salmonellosis in pigs with postweaning multisystemic wasting syndrome. Vet. Rec. 156, 583–4.

Haagmans, B.L., Osterhaus, A.D.M.E.M.E., 2006. Coronaviruses and their therapy. Antiviral Res. 71, 397–403.

Halaihel, N., Masía, R.M., Fernández-Jiménez, M., Ribes, J.M., Montava, R., De Blas, I., Gironés, O., Alonso, J.L., Buesa, J., 2010. Enteric calicivirus and rotavirus infections in domestic pigs. Epidemiol. Infect. 138, 542–8.

Halperin, A., Buhot, A., Zhulina, E.B., 2006. Hybridization at a surface: The role of spacers in DNA microarrays. Langmuir 22, 11290–11304.

Halter, M.R., Joens, L.A., 1988. Lipooligosaccharides from Treponema hyodysenteriae

and Treponema innocens. Infect. Immun. 56, 3152–6.

Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. 72, 5262–7.

Hammitt, M.C., Bueschel, D.M., Keel, M.K., Glock, R.D., Cuneo, P., DeYoung, D.W., Reggiardo, C., Trinh, H.T., Songer, J.G., 2008. A possible role for Clostridium difficile in the etiology of calf enteritis. Vet. Microbiol. 127, 343–52.

Hampson, D., Fellström, C., Thomson, J., 2006. Swine dysentery. In: Straw, B., Zimmerman, J., Dallaire, S., Taylor, D. (Eds.), Diseases of Swine. Blackwell, Ames, Iowa State, pp. 785–805.

Hampson, D.D.J., La, T., Phillips, N.D. 2015. Emergence of Brachyspira species and strains: reinforcing the need for surveillance. Porc. Heal. Manag. 1, 8.

Han, M.G., Smiley, J.R., Thomas, C., Saif, L.J., 2004. Genetic recombination between two genotypes of genogroup III bovine noroviruses (BoNVs) and capsid sequence diversity among BoNVs and Nebraska-like bovine enteric caliciviruses. J. Clin. Microbiol. 42, 5214–24.

Han, W., Liu, B., Cao, B., Beutin, L., Krüger, U., Liu, H., Li, Y., Liu, Y., Feng, L., Wang, L., 2007. DNA microarray-based identification of serogroups and virulence gene patterns of Escherichia coli isolates associated with porcine postweaning diarrhea and edema disease. Appl. Environ. Microbiol. 73, 4082–8.

Hanon, J.-B., Van der Stede, Y., Antonissen, A., Mullender, C., Tignon, M., van den Berg, T., Caij, B., 2014. Distinction between persistent and transient infection in a bovine viral diarrhoea (BVD) control programme: appropriate interpretation of real-time RT-PCR and antigen-ELISA test results. Transbound. Emerg. Dis. 61, 156–62.

Harding, J., Done, J., Kershaw, G., 1957. A transmissible polioencephalomyelitis of pigs (Talfan disease). Vet. Rec. 69, 824–832.

Harrington, C.R., Lucchini, S., Ridgway, K.P., Wegmann, U., Eaton, T.J., Hinton, J.C.D., Gasson, M.J., Narbad, A., 2008. A short-oligonucleotide microarray that allows improved detection of gastrointestinal tract microbial communities. BMC Microbiol. 8, 195.

Harris, N.B., Barletta, R.G., 2001. Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine. Clin. Microbiol. Rev.

Haschek, B., Klein, D., Benetka, V., Herrera, C., Sommerfeld-Stur, I., Vilcek, S., Moestl, K., Baumgartner, W., 2006. Detection of bovine torovirus in neonatal calf diarrhoea in Lower Austria and Styria (Austria). J. Vet. Med. B. Infect. Dis. Vet. Public Health 53, 160–5.

Hasoksuz, M., Lathrop, S., Al-dubaib, M.A., Lewis, P., Saif, L.J., 1999a. Antigenic variation among bovine enteric coronaviruses (BECV) and bovine respiratory coronaviruses (BRCV) detected using monoclonal antibodies. Arch. Virol. 144, 2441–7.

Hasoksuz, M., Lathrop, S.L., Gadfield, K.L., Saif, L.J., 1999b. Isolation of bovine respiratory coronaviruses from feedlot cattle and comparison of their biological and antigenic properties with bovine enteric coronaviruses. Am. J. Vet. Res. 60, 1227–33.

Hassine-Zaafrane, M., Kaplon, J., Sdiri-Loulizi, K., Aouni, Z., Pothier, P., Aouni, M., Ambert-Balay, K., 2012. Molecular prevalence of bovine noroviruses and neboviruses detected in central-eastern Tunisia. Arch. Virol. 157, 1599–604.

Hatheway, C.L., 1990. Toxigenic clostridia. Clin. Microbiol. Rev. 3, 66–98.

Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A., Malorny, B., 2011. Diversity of Salmonella enterica serovar Derby isolated from pig, pork and humans in Germany. Int. J. Food Microbiol. 151, 141–9.

Hayward, M.R., Jansen, V. a a, Woodward, M.J., 2013. Comparative genomics of Salmonella enterica serovars Derby and Mbandaka, two prevalent serovars associated with different livestock species in the UK. BMC Genomics 14, 365.

He, J.-L., Dai, D., Zhou, N., Zhou, J.-Y., 2012. Analysis of putative ORF3 gene within porcine circovirus type 2. Hybridoma (Larchmt). 31, 180–7.

He, X., Xu, X., Li, K., Liu, B., Yue, T., 2016. Identification of Salmonella enterica Typhimurium and variants using a novel multiplex PCR assay. Food Control 65, 152–159.

He, Z., Wu, L., Li, X., Fields, M.W., Zhou, J., 2005. Empirical Establishment of Oligonucleotide Probe Design Criteria. Appl. Environ. Microbiol. 71, 3753–3760.

Heath, L., van der Walt, E., Varsani, A., Martin, D.P., 2006. Recombination patterns in aphthoviruses mirror those found in other picornaviruses. J. Virol. 80, 11827–11832.

Hecker, K.H., Roux, K.H., 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. Biotechniques 20, 478–85.

Heffron, F., Niemann, G., Yoon, H., Kidwai, A., Brown, R., McDermott, J., Smith, R., Adkins, J., 2011. Salmonella-secreted virulence factors. In: Porwollik, S. (Ed.), Salmonella : From Genome to Function. Horizon Scientific Press, p. 187.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–94.

Henihan, G., Schulze, H., Corrigan, D.K., Giraud, G., Terry, J.G., Hardie, A., Campbell, C.J., Walton, A.J., Crain, J., Pethig, R., Templeton, K.E., Mount, A.R., Bachmann, T.T., 2016. Label- and amplification-free electrochemical detection of bacterial ribosomal RNA. Biosens. Bioelectron. 81, 487–494.

Hermann-Bank, M.L., Skovgaard, K., Stockmarr, A., Strube, M.L., Larsen, N., Kongsted, H., Ingerslev, H.-C., Mølbak, L., Boye, M.. 2015. Characterization of the bacterial gut microbiota of piglets suffering from new neonatal porcine diarrhoea. BMC Vet. Res. 11, 139.

Hernandez, G.E., Mondala, T.S., Head, S.R., 2009. Assessing a novel room-temperature RNA storage medium for compatibility in microarray gene expression analysis. Biotechniques 47, 667–670.

Hert, D.G., Fredlake, C.P., Barron, A.E., 2008. Advantages and limitations of next-generation sequencing technologies: a comparison of electrophoresis and non-electrophoresis methods. Electrophoresis 29, 4618–26.

Hicks, S.C., Irizarry, R.A., 2014. When to use Quantile Normalization? bioRxiv 012203.

Hill, D.L., Yang, T.-J. (Thomas), 1984. Virus-like particles in a positive case of canine parvovirus enteritis. Micron Microsc. Acta 15, 207–209.

Hilton, S.K., Castro-Nallar, E., Pérez-Losada, M., Toma, I., McCaffrey, T.A., Hoffman, E.P., Siegel, M.O., Simon, G.L., Johnson, W.E., Crandall, K.A., 2016. Metataxonomic and Metagenomic Approaches vs. Culture-Based Techniques for Clinical Pathology. Front. Microbiol. 7, 484.

Hodges, K., Gill, R., 2010. Infectious diarrhea: Cellular and molecular mechanisms. Gut Microbes 1, 4–21.

Hoet, A., Nielsen, P., Hasoksuz, M., Thomas, C., Wittum, T., Saif, L., 2003. Detection of Bovine Torovirus and other Enteric Pathogens in Feces from Diarrhea Cases in Cattle. J. Vet. Diagnostic Investig. 15, 205–212.

Hoet, A.E., Saif, L.J., 2004. Bovine torovirus (Breda virus) revisited. Anim. Health Res. Rev. 5, 157–71.

Hoet, A.E., Smiley, J., Thomas, C., Nielsen, P.R., Wittum, T.E., Saif, L.J., 2003. Association of enteric shedding of bovine torovirus (Breda virus) and other enteropathogens with diarrhea in veal calves. Am. J. Vet. Res. 64, 485–90.

Holyoake, P., Emery, D., Gonsalves, J., Donahoo, M., Collins, A., 2010. Prevalence of antibodies to *Lawsonia intracellularis* in pig herds in Australia. Aust. Vet. J. 88, 186–188.

HONDA, E., KIMATA, A., HATTORI, I., KUMAGAI, T., TSUDA, T., TOKUI, T., 1990. A Serological Comparison of 4 Japanese Isolates of Porcine Enteroviruses with the

International Reference Strains. Japanese J. Vet. Sci. 52, 49–54.

Hong, B.X., Jiang, L.F., Hu, Y.S., Fang, D.Y., Guo, H.Y., 2004. Application of oligonucleotide array technology for the rapid detection of pathogenic bacteria of foodborne infections. J. Microbiol. Methods 58, 403–411.

Horton, J., 2014. Helminth-Nematode: Trichuris trichiura. In: Motarjemi, Y., Moy, G., Todd, E. (Eds.), Encyclopedia of Food Safety. Academic Press Elsevier, pp. 111–115.

Hoshino, Y., Kapikian, A.Z., 2000. Rotavirus Serotypes : Classification and Importance in Epidemiology , Immunity , and Vaccine Development. J. Health. Popul. Nutr. 18, 5–14.

Hosmillo, M.D.T., Jeong, Y.-J., Kim, H.-J., Collantes, T.M., Alfajaro, M.M., Park, J.-G., Kim, H.-H., Kwon, H.-J., Park, S.-J., Kang, M.-I., Park, S.-I., Cho, K.-O., 2010. Development of universal SYBR Green real-time RT-PCR for the rapid detection and quantitation of bovine and porcine toroviruses. J. Virol. Methods 168, 212–7.

Houe H, 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microbiol. 64, 89 –107.

Houe H and Meyling A. 1991. Prevalence of bovine virus diarrhoea (BVD) in 19 Danish dairy herds and estimation of incidence of infection in early pregnancy. Prev. Vet. Med. 11: 9-16.

House, J.K., Smith, B.P., Dilling, G.W., Roden, L.D., 1993. Enzyme-linked immunosorbent assay for serologic detection of Salmonella dublin carriers on a large dairy. Am. J. Vet. Res. 54, 1391–9.

Howlett, S.E., Castillo, H.S., Gioeni, L.J., Robertson, J.M., Donfack, J., 2014. Evaluation of DNAstable^{TM} for DNA storage at ambient temperature. Forensic Sci. Int. Genet. 8, 170–178.

Huang, Y.T, Yang, J.I, Chrobak, M., Borneman, J., 2014. PRISE2: Software for designing sequence-selective PCR primers and probes. BMC Bioinformatics. 15: 317-325.

Hudson, M.J., Alexander, T.J., Lysons, R.J., 1976. Diagnosis of swine dysentery: spirochaetes which may be confused with Treponema hyodysenteriae. Vet. Rec. 99, 498–500.

Hughes, T.R., Mao, M., Jones, A.R., Burchard, J., Marton, M.J., Shannon, K.W., Lefkowitz, S.M., Ziman, M., Schelter, J.M., Meyer, M.R., Kobayashi, S., Davis, C., Dai, H., He, Y.D., Stephaniants, S.B., Cavet, G., Walker, W.L., West, A., Coffey, E., Shoemaker, D.D., Stoughton, R., Blanchard, A.P., Friend, S.H., Linsley, P.S., 2001. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat. Biotechnol. 19, 342–7.

Hur, T.Y., Jung, Y.H., Choe, C.Y., Cho, Y. Il, Kang, S.J., Lee, H.J., Ki, K.S., Baek, K.S., Suh, G.H., 2013. The dairy calf mortality : the causes of calf death during ten years at a large dairy farm in Korea. Korean J. Vet. Res. 53, 103–108.

ICTV, 2008. International Committee of Taxonomy in Virology. Discussion forum of the International Committee on Taxonomy of Viruses.

Ito, T., Okada, N., Fukuyama, S., 2007. Epidemiological analysis of bovine torovirus in Japan. Virus Res. 126, 32–37.

Ivanova, N. V., Kuzmina, M.L., 2013. Protocols for dry DNA storage and shipment at room temperature. Mol. Ecol. Resour. 13, 890–898.

Jaakkola, K., Somervuo, P., Korkeala, H., 2015. Comparative genomic hybridization analysis of yersinia enterocolitica and yersinia pseudotuberculosis identifies genetic traits to elucidate their different ecologies. Biomed Res. Int. 2015.

Jääskeläinen, A.J., Piiparinen, H., Lappalainen, M., Koskiniemi, M., Vaheri, A., 2006. Multiplex-PCR and oligonucleotide microarray for detection of eight different herpesviruses from clinical specimens. J. Clin. Virol. 37, 83–90.

Jackson, R., Morris, D.J., Cooper, R.J., Bailey, A.S., Klapper, P.E., Cleator, G.M., Tullo, A.B., 1996. Multiplex polymerase chain reaction for adenovirus and herpes simplex virus in eye swabs. J. Virol. Methods 56, 41–48.

Jacobs, B.L., Samuel, C.E., 1985. Biosynthesis of reovirus-specified polypeptides: the reovirus s1 mRNA encodes two primary translation products. Virology 143, 63–74.

Jacobson, M., Andersson, M., Lindberg, R., Fossum, C., Jensen-Waern, M., 2011. Microarray and cytokine analyses of field cases of pigs with diarrhoea. Vet. Microbiol. 153, 307–14.

Järvinen, A.-K., Laakso, S., Piiparinen, P., Aittakorpi, A., Lindfors, M., Huopaniemi, L., Piiparinen, H., Mäki, M., 2009. Rapid identification of bacterial pathogens using a PCR- and microarray-based assay. BMC Microbiol. 9, 161.

Jayaraman, A., Hall, C.K., Genzer, J., 2007. Computer simulation study of probe-target hybridization in model DNA microarrays: effect of probe surface density and target concentration. J. Chem. Phys. 127, 144912.

Jenkins, C., Tembo, M., Chart, H., Cheasty, T., Willshaw, G.A., Phillips, A.D., Tompkins, D., Smith, H., 2006. Detection of enteroaggregative Escherichia coli in faecal samples from patients in the community with diarrhoea. J. Med. Microbiol. 55, 1493–1497.

Jensen, Ê., Strodl, J., Frydendahl, K., Ka, T., Fredholm, M., Evans, G., Kåre Jensen, T., Strodl Andersen, J., Fredholm, M., Evans, G., 2003. Association between the

porcine Escherichia coli F18 receptor genotype and phenotype and susceptibility to colonisation and postweaning diarrhoea caused by E. coli O138:F18. Vet. Microbiol. 93, 39–51.

Jensen, N.S., Stanton, T.B., 1993. Comparison of Serpulina hyodysenteriae B78, the type strain of the species, with other S. hyodysenteriae strains using enteropathogenicity studies and restriction fragment length polymorphism analysis. Vet. Microbiol. 36, 221–31.

Jensen, T.K.K., Vigre, H., Svensmark, B., Bille-Hansen, V., 2006. Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by Lawsonia intracellularis. J. Comp. Pathol. 135, 176–82.

Jeong, J.-H., Kim, G.-Y., Yoon, S.-S., Park, S.-J., Kim, Y.-J., Sung, C.-M., Jang, O.-J., Shin, S.-S., Koh, H.-B., Lee, B.-J., Lee, C.-Y., Kang, M.-I., Kim, H.-J., Park, N.-Y., Cho, K.-O., 2005. Detection and isolation of winter dysentery bovine coronavirus circulated in Korea during 2002-2004. J. Vet. Med. Sci. 67, 187–9.

Jeong, Y.-J., Park, S.-J.S.-I., Hosmillo, M., Shin, D.-J., Chun, Y.-H., Kim, H.-J., Kwon, H.-J., Kang, S.-Y., Woo, S.-K., Park, S.-J.S.-I., Kim, G.-Y., Kang, M.-I., Cho, K.-O., 2009. Detection and molecular characterization of porcine group C rotaviruses in South Korea. Vet. Microbiol. 138, 217–24.

Jeoung, H.-Y., Lim, J.-A., Jeong, W., Oem, J.-K., An, D.-J., 2011. Three clusters of bovine kobuvirus isolated in Korea, 2008-2010. Virus Genes 42, 402–6.

Jex, A.R., Stanley, K.K., Lo, W., Littman, R., Verweij, J.J., Campbell, B.E., Nolan, M.J., Pangasa, A., Stevens, M.A., Haydon, S., Gasser, R.B., 2012. Detection of diarrhoeal pathogens in human faeces using an automated, robotic platform. Mol. Cell. Probes 26, 11–15.

Jiang X., Wang M., Wang K., E.M.K., 1993. Sequence and genomic organization of Norwalk virus. Virology 195, 51–61.

Jiang, X., Huang, P.W., Zhong, W.M., Farkas, T., Cubitt, D.W., Matson, D.O., 1999. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J. Virol. Methods 83, 145–54.

Jiang, Y., Guo, Y., Wang, P., Dong, Q., Opriessnig, T., Cheng, J., Xu, H., Ding, X., Guo, J., 2011. A novel diagnostic platform based on multiplex ligase detection–PCR and microarray for simultaneous detection of swine viruses. J. Virol. Methods 178, 171–178.

Jiang, Y., Shang, H., Xu, H., Ding, X., Zhao, L., Fang, L., Chen, W., 2010. Detection and genotyping of porcine circovirus in naturally infected pigs by oligo-microarray. Res. Vet. Sci. 89, 133–139.

Jinghui, F., Yijing, L., 2005. Cloning and sequence analysis of the M gene of porcine

epidemic diarrhea virus LJB/03. Virus Genes 30, 69–73.

Joachim, A., Ruttkowski, B., Zimmermann, M., Daugschies, A., Mundt, H.-C., 2004. Detection of Isospora suis (Biester and Murray 1934) in piglet faeces--comparison of microscopy and PCR. J. Vet. Med. B. Infect. Dis. Vet. Public Health 51, 140–2.

Jolley, W.R., Bardsley, K.D., 2006. Ruminant Coccidiosis. Vet. Clin. North Am. Food Anim. Pract. 22, 613–621.

Jonach, B., Boye, M., Stockmarr, A. 2014. Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea. BMC Vet. Res. 10, 68.

Jordan, E.K., Sever, J.L., 1994. Fetal damage caused by parvoviral infections. Reprod. Toxicol. 8, 161–189.

Jothikumar, N., Griffiths, M.W., 2002. Rapid detection of Escherichia coli O157:H7 with multiplex real-time PCR assays. Appl Env. Microbiol 68, 3169–3171.

Jufare, A., Awol, N., Tadesse, F., Tsegaye, Y., Hadush, B., 2015. Parasites of pigs in two farms with poor husbandry practices in Bishoftu, Ethiopia. Onderstepoort J. Vet. Res. 82, 839.

Juhas, M., Van Der Meer, J.R., Gaillard, M., Harding, R.M., Hood, D.W., Crook, D.W., 2009. Genomic islands: Tools of bacterial horizontal gene transfer and evolution. FEMS Microbiol. Rev.

Jung, K., Kang, B.K., Lee, C.S., Song, D.S., 2008. Impact of porcine group A rotavirus co-infection on porcine epidemic diarrhea virus pathogenicity in piglets. Res Vet Sci 84, 502–506.

Kaewpongsri, S., Sukasem, C., Srichunrusami, C., Pasomsub, E., Zwang, J., Pairoj, W., Chantratita, W., 2010. An integrated bioinformatics approach to the characterization of influenza A/H5N1 viral sequences by microarray data: Implication for monitoring H5N1 emerging strains and designing appropriate influenza vaccines. Mol. Cell. Probes 24, 387–395.

Kaku, Y., Sarai, A., Murakami, Y., 2001. Genetic reclassification of porcine enteroviruses. J. Gen. Virol. 82, 417–24.

Kane, M.D., Jatkoe, T.A., Stumpf, C.R., Lu, J., Thomas, J.D., Madore, S.J., 2000. Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays 28, 4552–4557.

Kang, S.. J., Ryu, S.. J., Chae, J.. S., Eo, S.. K., Woo, G.. J., Lee, J.. H., 2004. Occurrence and characteristics of enterohemorrhagic Escherichia coli O157 in calves associated with diarrhoea. Vet. Microbiol. 98, 323–328.

Kapikian, A., Chanock, R., 1996. Rotaviruses. In: Fields, B., Knipe, D., Howley, P. (Eds.), Fields Virology. Lippincott-Raven Press, Philadelphia, pp. 1657–1708.

Kaplon, J.J., Guenau, E., Asdrubal, P., Pothier, P., Ambert-Balay, K., 2011. Possible novel Nebovirus genotype in cattle, France. Emerg. Infect. Dis. 17, 1120–1123.

Karamon, J., Ziomko, I., Cencek, T., 2007. Prevalence of Isospora suis and Eimeria spp. in suckling piglets and sows in Poland, Veterinary Parasitology.

Kasza, L., 1970. Isolation and characterisation of a reovirus from pigs. Vet. Rec. 87, 681–6.

Kato, N., Ou, C.-Y., Kato, H., Bartley, S.L., Brown, V.K., Dowell, V.R., Kazue, A., 1991. Identification of Toxigenic Clostridium dijficile by the Polymerase Chain Reaction. J. Clin. Microbiol. 33–37.

Kawashima, K., Katsuda, K., Tsunemitsu, H., 2007. Epidemiological investigation of the prevalence and features of postweaning multisystemic wasting syndrome in Japan. J. Vet. Diagn. Invest. 19, 60–8.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I., Moore, R.J., 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS Pathog. 4, e26.

Khamrin, P., Maneekarn, N., Hidaka, S., Kishikawa, S., Ushijima, K., Okitsu, S., Ushijima, H., 2010. Molecular detection of kobuvirus sequences in stool samples collected from healthy pigs in Japan. Infect. Genet. Evol. 10, 950–954.

Khamrin, P., Maneekarn, N., Kongkaew, A., Kongkaew, S., Okitsu, S., Ushijima, H., 2009. Porcine kobuvirus in piglets, Thailand. Emerg. Infect. Dis. 15, 2075–6.

Khamrin, P., Maneekarn, N., Peerakome, S., Okitsu, S., Mizuguchi, M., Ushijima, H., 2008. Bovine kobuviruses from cattle with diarrhea. Emerg. Infect. Dis. 14, 985–6.

Khamrin, P., Okame, M., Thongprachum, A., Nantachit, N., Nishimura, S., Okitsu, S., Maneekarn, N., Ushijima, H., 2011. A single-tube multiplex PCR for rapid detection in feces of 10 viruses causing diarrhea. J. Virol. Methods 173, 390–393.

Khan, S.M., Debnath, C., Pramanik, A.K., Xiao, L., Nozaki, T., Ganguly, S., 2010. Molecular characterization and assessment of zoonotic transmission of Cryptosporidium from dairy cattle in West Bengal, India. Vet. Parasitol. 171, 41–47.

Khelef, D., Saïb, M.Z., Akam, A., Kaidi, R., Chirila, V., Cozma, V., Adjou, E.K.T., 2007. Épidemiologie de la cryptosporidiose chez les bovins en Algérie 158, 260–264.

Kibbe, W.A., 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35, W43–6.

Kikuchi, E., Miyamoto, Y., Narushima, S., Itoh, K., 2002. Design of species-specific primers to identify 13 species of Clostridium harbored in human intestinal tracts. Microbiol. Immunol. 46, 353–8.

Kim, H.J., Cho, H.S., Cho, K.O., Park, N.Y., 2006. Detection and molecular characterization of porcine enteric calicivirus in Korea, genetically related to sapoviruses. J. Vet. Med. B. Infect. Dis. Vet. Public Health 53, 155–9.

Kim, M., Oh, H.S., Park, S.C., Chun, J., 2014. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int. J. Syst. Evol. Microbiol. 64, 346–351.

Kim, O., Chae, C., 2000. In Situ Hybridization for the Detection and Localization of Porcine Epidemic Diarrhea Virus in the Intestinal Tissues from Naturally Infected Piglets. Vet. Pathol. 37, 62–67.

Kim, O., Kim, B., Chae, C., 1998. Prevalence of Lawsonia intracellularis in selected pig herds in Korea as determined by PCR. Vet. Rec. 143, 587–9.

Kim, S.-H., Kim, I.-J., Pyo, H.-M., Tark, D.-S., Song, J.-Y., Hyun, B.-H., 2007. Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. J. Virol. Methods 146, 172–177.

King, A., Brown, F., Christian, P., Hovi, T., Hyypiä, T., Knowles, N., Lemon, S., Minor, P., Palmenberg, A., Skern, T., Stanway, G., 1999. Picornaviridae. In: Van Regenmortel, M., Fauquet, C., Bishop, D., Calisher, C., Carsten, E., Estes, M., Lemon, S., Maniloff, J., Mayo, M., McGeoch, D., Pringle, C., Wickner, R. (Eds.), In Virus Taxonomy - Seventh Report of the International Committee for the Taxonomy of Viruses. Academic Press, New York, NY, p. 996.

Kirisawa, R., Takeyama, A., Koiwa, M., Iwai, H., 2007. Detection of Bovine Torovirus in Fecal Specimens of Calves with Diarrhea in Japan. J. Vet. Med. Sci. 69, 471–476.

Kistler, A., Avila, P.C., Rouskin, S., Wang, D., Ward, T., Yagi, S., Schnurr, D., Ganem, D., DeRisi, J.L., Boushey, H.A., 2007. Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. J. Infect. Dis. 196, 817–25.

Klein, D., Alispahic, M., Sofka, D., Iwersen, M., Drillich, M., Hilbert, F., 2013. Prevalence and risk factors for shedding of thermophilic Campylobacter in calves with and without diarrhea in Austrian dairy herds. J. Dairy Sci. 96, 1203–1210.

Knowles, NJ, Hovi,T, Hyypiä, T, King,AMQ, Lindberg, AM, Pallansch, MA, Palmenberg AC, Simmonds, P, Skern, T, Stanway,G, Yamashita, T, Zell, R., 2012. Picornaviridae. In: King, AMQ, Adams, MJ, Carstens, EB, Lefkowitz, E. (Ed.), Virus Taxonomy: Classification and Nomenclature of Viruses: . Elsevier, San Diego, pp.

855–880.

Knowles, N., 2005. Genetic relationships between bovine enteroviruses inVP1 and 3Dpol. In: EUROPIC 2005 - XIIIth Meet. Eur. Study Group Mol. Biol. Picornaviridae. Lunteren, The Netherlands, p. A19.

Knowles, N., 2006. Porcine enteric picornaviruses. In: Straw, B., Zimmermann, J., D'Allaire, S., Taylor, D. (Eds.), Diseases of Swine. Blackwell Publishing Inc., Ames, pp. 337–345.

Knowles, N.J., Barnett, I.T.R., 1985. A serological classification of bovine enteroviruses. Arch. Virol. 83, 141–155.

Knowles, N.J., Buckley, L.S., Pereira, H.G., 1979. Classification of porcine enteroviruses by antigenic analysis and cytopathic effects in tissue culture: Description of 3 new serotypes. Arch. Virol. 62, 201–208.

Kolenda, R., Burdukiewicz, M., Schierack, P., 2015. A systematic review and meta-analysis of the epidemiology of pathogenic Escherichia coli of calves and the role of calves as reservoirs for human pathogenic E. coli. Front. Cell. Infect. Microbiol. 5, 23.

Koltai, H., Weingarten-Baror, C., 2008. Specificity of DNA microarray hybridization: characterization, effectors and approaches for data correction. Nucleic Acids Res. 36, 2395–405.

Koonin, E. V, Dolja, V. V, 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol. Biol. 28, 375–430.

Koressaar T. and Remm M. 2012. Characterization of species-specific repeats in 613 prokaryotic species. DNA Res. 19(3): 219–230.

Korkola, J.E., Estep, A.L.H., Pejavar, S., DeVries, S., Jensen, R., Waldman, F.M., 2003. Optimizing stringency for expression microarrays. Biotechniques 35, 828–835.

Kotilainen, P., Jalava, J., Meurman, O., Lehtonen, O.P., Rintala, E., Seppälä, O.P., Eerola, E., Nikkari, S., 1998. Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. J. Clin. Microbiol. 36, 2205–9.

Koutny, H., Joachim, A., Tichy, A., Baumgartner, W., 2012. Bovine Eimeria species in Austria. Parasitol. Res. 110, 1893–901.

Krumbholz, A., Dauber, M., Henke, A., Birch-hirschfeld, E., Knowles, N.J., Stelzner, A., Zell, R., 2002. Sequencing of porcine enterovirus groups II and III reveals unique features of both virus groups. J Virol 76, 5813–5821.

Krutkiewicz, A., Klimuszko, D., 2010. Genotyping and PCR detection of potential virulence genes in Campylobacter jejuni and Campylobacter coli isolates from

different sources in Poland. Folia Microbiol. (Praha). 55, 167–175.

Ku, W.C., Lau, W.K., Tseng, Y.T., Tzeng, C.M., Chiu, S.K., 2004. Dextran sulfate provides a quantitative and quick microarray hybridization reaction. Biochem. Biophys. Res. Commun. 315, 30–37.

Kuehne, S.A., Cartman, S.T., Heap, J.T., Kelly, M.L., Cockayne, A., Minton, N.P., 2010. The role of toxin A and toxin B in Clostridium difficile infection. Nature 467, 711–714.

Kuhn, H., Demidov, V. V., Coull, J.M., Fiandaca, M.J., Gildea, B.D., Frank-Kamenetskii, M.D., 2002. Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J. Am. Chem. Soc. 124, 1097–1103.

Kváč, M., Kestřánová, M., Pinková, M., Květoňová, D., Kalinová, J., Wagnerová, P., Kotková, M., Vítovec, J., Ditrich, O., McEvoy, J., Stenger, B., Sak, B., 2013. Cryptosporidium scrofarum n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (Sus scrofa). Vet. Parasitol. 191, 218–27.

Kwon, H.-J., Kim, H.-H., Kim, H.-J., Park, J.-G., Son, K.-Y., Jung, J., Lee, W.S., Cho, K.-O., Park, S.-J., Kang, M.-I., 2012. Detection and molecular characterization of porcine type 3 orthoreoviruses circulating in South Korea. Vet. Microbiol. 157, 456–63.

La Rosa, G., Muscillo, M., Di Grazia, A., Fontana, S., Iaconelli, M., Tollis, M., 2006. Validation of rt-PCR assays for molecular characterization of porcine teschoviruses and enteroviruses. J. Vet. Med. B. Infect. Dis. Vet. Public Health 53, 257–65.

La, T., Phillips, N.D., Wanchanthuek, P., Bellgard, M.I., O'Hara, A.J., Hampson, D.J., 2011. Evidence that the 36 kb plasmid of Brachyspira hyodysenteriae contributes to virulence. Vet. Microbiol. 153, 150–5.

Lai M.M.C., H.K.V., 2001. Coronaviridae. In: M, K.D.M.& . H.P. (Ed.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1163–1185.

Lam, H.Y.K., Clark, M.J., Chen, R.R., Chen, R.R., Natsoulis, G., O'Huallachain, M., Dewey, F.E., Habegger, L., Ashley, E. a, Gerstein, M.B., Butte, A.J., Ji, H.P., Snyder, M., 2012. Performance comparison of whole-genome sequencing platforms. Nat. Biotechnol. 30, 562–562.

Lan, D., Ji, W., Yang, S., Cui, L., Yang, Z., Yuan, C., Hua, X., 2011. Isolation and characterization of the first Chinese porcine sapelovirus strain. Arch. Virol. 156, 1567–74.

Lane, S., Evermann, J., Loge, F., Call, D.R., 2004. Amplicon secondary structure prevents target hybridization to oligonucleotide microarrays. Biosens. Bioelectron. 20, 728–735.

Langpap, T.J., Bergeland, M.E., Reed, D.E., 1979. Coronaviral enteritis of young calves: virologic and pathologic findings in naturally occurring infections. Am. J. Vet. Res. 40, 1476–8.

Langridge, G., Wain, J., Nair, S., 2008. Invasive Salmonellosis in Humans. In: Böck, A., Curtiss III, R., Kaper, J., PD, K., Neidhardt, F., Nyström, T., Slauch, J., Squires, C., Ussery, D. (Eds.), EcoSal- Escherichia Coli and Salmonella. Cell. Mol. Biol. ASM Press, Washington DC.

Lappin, M.R., 2009. Infectious Disease Diagnostic Assays. Top. Companion Anim. Med. 24, 199–208.

Larsson, J., Jacobson, M., Fall, N., Båverud, V., Grandon, R., Lindberg, R., Aspán, A., 2015. Pathological and bacteriological characterization of neonatal porcine diarrhoea of uncertain aetiology. J. Med. Microbiol. 64, 916–926.

Larsson, J., Lindberg, R., Aspán, A., Grandon, R., Westergren, E., Jacobson, M., 2014. Neonatal Piglet Diarrhoea Associated with Enteroadherent Enterococcus hirae. J. Comp. Pathol. 151, 137–147.

Lasek-Nesselquist, E., Welch, D.M., Sogin, M.L., 2010. The identification of a new Giardia duodenalis assemblage in marine vertebrates and a preliminary analysis of G. duodenalis population biology in marine systems. Int. J. Parasitol. 40, 1063–1074.

Lau, S.K.P., Li, K.S.M., Huang, Y., Shek, C.-T., Tse, H., Wang, M., Choi, G.K.Y., Xu, H., Lam, C.S.F., Guo, R., Chan, K.-H., Zheng, B.-J., Woo, P.C.Y., Yuen, K.-Y., 2010. Ecoepidemiology and complete genome comparison of different strains of severe acute respiratory syndrome-related Rhinolophus bat coronavirus in China reveal bats as a reservoir for acute, self-limiting infection that allows recombination events. J. Virol. 84, 2808–19.

Laude, H., Van Reeth, K., Pensaert, M., 1993. Porcine respiratory coronavirus: molecular features and virus-host interactions. Vet. Res. 24, 125–50.

Lazcka, O., Campo, F.J. Del, Muñoz, F.X., 2007. Pathogen detection: A perspective of traditional methods and biosensors. Biosens. Bioelectron. 22, 1205–1217.

LeBlanc, N., Gantelius, J., Schwenk, J.M., Ståhl, K., Blomberg, J., Andersson-Svahn, H., Belák, S., 2009. Development of a magnetic bead microarray for simultaneous and simple detection of four pestiviruses. J. Virol. Methods 155, 1–9.

LeBlanc, N., Leijon, M., Jobs, M., Blomberg, J., Belák, S., 2010. A novel combination of TaqMan RT-PCR and a suspension microarray assay for the detection and species identification of pestiviruses. Vet. Microbiol. 142, 81–86.

Lebrun, M., Filée, P., Mousset, B., Desmecht, D., Galleni, M., Mainil, J.G., Linden, A., 2007. The expression of Clostridium perfringens consensus beta2 toxin is

associated with bovine enterotoxaemia syndrome. Vet. Microbiol. 120, 151–157.

Lee, C.S., Lee, J., 2010. Evaluation of new gyrB-based real-time PCR system for the detection of B. fragilis as an indicator of human-specific fecal contamination. J. Microbiol. Methods 82, 311–8.

Lee, I., Dombkowski, A.A., Athey, B.D., 2004. Guidelines for incorporating non-perfectly matched oligonucleotides into target-specific hybridization probes for a DNA microarray. Nucleic Acids Res. 32, 681–690.

Lee, S.B., Clabaugh, K.C., Silva, B., Odigie, K.O., Coble, M.D., Loreille, O., Scheible, M., Fourney, R.M., Stevens, J., Carmody, G.R., Parsons, T.J., Pozder, A., Eisenberg, A.J., Budowle, B., Ahmad, T., Miller, R.W., Crouse, C.A., 2012. Assessing a novel room temperature DNA storage medium for forensic biological samples. Forensic Sci. Int. Genet. 6, 31–40.

Lee, Y.F., Nomoto, A., Detjen, B.M., Wimmer, E., 1977. A protein covalently linked to poliovirus genome RNA. Proc. Natl. Acad. Sci. U. S. A. 74, 59–63.

Lefterova, M.I., Suarez, C.J., Banaei, N., Pinsky, B.A., 2015. Next-Generation Sequencing for Infectious Disease Diagnosis and Management: A Report of the Association for Molecular Pathology, The Journal of Molecular Diagnostics.

Leinberger, D.M., Schumacher, U., Autenrieth, I.B., Bachmann, T.T., 2005. Development of a DNA microarray for detection and identification of fungal pathogens involved in invasive mycoses. J. Clin. Microbiol. 43, 4943–4953.

Lemoine, S., Combes, F., Le Crom, S., 2009. An evaluation of custom microarray applications: the oligonucleotide design challenge. Nucleic Acids Res. 37, 1726–39.

Leroy, Q., Raoult, D., 2010. Review of microarray studies for host–intracellular pathogen interactions. J. Microbiol. Methods 81, 81–95.

Letellier, C., Kerkhofs, P., 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. J. Virol. Methods 114, 21–27.

Letowski, J., Brousseau, R., Masson, L., 2004. Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. J. Microbiol. Methods 57, 269–78.

Levine, M.M., 1987. Escherichia coli that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 155, 377–89.

Ley, V., Higgins, J., Fayer, R., 2002. Bovine enteroviruses as indicators of fecal contamination. Appl. Environ. Microbiol. 68, 3455–3461.

Li, B., Ma, J., Xiao, S., Wen, L., Ni, Y., Zhang, X., Fang, L., He, K., 2012. Genome Sequence

of a Highly Prevalent Porcine Partetravirus in Mainland China. J. Virol. 86, 1899–1899.

Li, B., Ma, J., Xiao, S., Wen, L., Ni, Y., Zhang, X., Fang, L., He, K., 2012. Genome Sequence of a Highly Prevalent Porcine Partetravirus in Mainland China. J. Virol. 86, 1899–1899.

Li, B., Ma, J.J., Xiao, S.B., Zhang, X.H., Wen, L.B., Mao, L., Ni, Y.X., Guo, R.L., Zhou, J.M., Lv, L.X., He, K.W., 2012. Development of a loop-mediated isothermal amplification method for rapid detection of porcine boca-like virus. J Virol Methods 179, 390–395.

Li, B., Xiao, S., Ma, J., Liu, Y., Mao, L., Wen, L., Mao, A., Zhang, X., Ni, Y., Guo, R., Zhou, J., Yu, Z., Lv, L., Wang, X., Fang, L., Chen, H., He, K., 2011. Development of a novel TaqMan-based real-time PCR assay for the detection of porcine boca-like virus (Pbo-likeV). Virol. J. 8, 357.

Li, E.S.Y., Ng, J.K.K., Wu, J.H., Liu, W.T., 2004. Evaluating single-base-pair discriminating capability of planar oligonucleotide microchips using a non-equilibrium dissociation approach. Environ. Microbiol. 6, 1197–1202.

Li, J., Adams, V., Bannam, T.L., Miyamoto, K., Garcia, J.P., Uzal, F.A., Rood, J.I., McClane, B.A., 2013. Toxin plasmids of Clostridium perfringens. Microbiol. Mol. Biol. Rev. 77, 208–33.

Li, R.W., Wu, S., Li, W., Navarro, K., Couch, R.D., Hill, D., Urban, J.F., 2012. Alterations in the porcine colon microbiota induced by the gastrointestinal nematode Trichuris suis. Infect. Immun. 80, 2150–7.

Li, W., Li, H., Liu, Y., Pan, Y., Deng, F., Song, Y., Tang, X., He, Q., 2012. New variants of porcine epidemic diarrhea virus, China, 2011. Emerg. Infect. Dis. 18, 1350–3.

Li, X., Zhou, Y., Ji, H., Xu, Z., Zhu, L., 2014. One-step reverse transcription-loop-mediated isothermal amplification assay for sensitive and rapid detection of porcine kobuvirus. J. Virol. Methods 207, 1–5.

Li, Y., Chang, J., Wang, Q., Yu, L., 2012. Isolation of two Chinese bovine enteroviruses and sequence analysis of their complete genomes. Arch Virol 157, 2369–2375.

Libby, S.J., Adams, L.G., Ficht, T.A., Allen, C., Whitford, H.A., Buchmeier, N.A., Bossie, S., Guiney, D.G., 1997. The spy genes on the Salmonella dublin virulence plasmid are required for severe enteritis and systemic infection in the natural host. Infect. Immun. 65, 1786–1792.

Lin, B., Wang, Z., Vora, G.J., Thornton, J.A., Schnur, J.M., Thach, D.C., Blaney, K.M., Ligler, A.G., Malanoski, A.P., Santiago, J., Walter, E.A., Agan, B.K., Metzgar, D., Seto, D., Daum, L.T., Kruzelock, R., Rowley, R.K., Hanson, E.H., Tibbetts, C., Stenger, D.A., 2006. Broad-spectrum respiratory tract pathogen identification

using resequencing DNA microarrays. Genome Res. 16, 527–535.

Lin, X.-Q., O'Reilly, K.L., Storz, J., 2002. Antibody responses of cattle with respiratory coronavirus infections during pathogenesis of shipping fever pneumonia are lower with antigens of enteric strains than with those of a respiratory strain. Clin. Diagn. Lab. Immunol. 9, 1010–3.

Lin, Y., Huang, Y., Yun, S., Lin, F., Huang, T., Jong, M., Chang, W., Huang, C., 2010. P . 572 The distribution and genotyping of porcine teschovirus in Taiwan 69, 6315–6315.

Lindroos, K., Sigurdsson, S., Johansson, K., Rönnblom, L., Syvänen, A.-C., 2002. Multiplex SNP genotyping in pooled DNA samples by a four-colour microarray system. Nucleic Acids Res. 30, e70.

Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R., Blagburn, B.L., 2000. Cryptosporidium andersoni n. sp. (Apicomplexa: Cryptosporiidae) from cattle, Bos taurus. J. Eukaryot. Microbiol. 47, 91–5.

Linko, V. and Dietz, H. 2013. The enabled state of DNA nanotechnology. Curr.Opin. Biotechnol. 24:555-561.

Lisby, G., 1998. Application of nucleic acid amplification in clinical microbiology. Methods Mol. Biol. 92, 1–29.

Litrup, E., Torpdahl, M., Malorny, B., Huehn, S., Christensen, H., Nielsen, E.M., 2010. Association between phylogeny, virulence potential and serovars of Salmonella enterica. Infect. Genet. Evol. 10, 1132–1139.

Liu, B., Wu, F., Li, D., Beutin, L., Chen, M., Cao, B., Wang, L., 2010. Development of a serogroup-specific DNA microarray for identification of Escherichia coli strains associated with bovine septicemia and diarrhea. Vet. Microbiol. 142, 373–378.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., Law, M., 2012. Comparison of next-generation sequencing systems. J. Biomed. Biotechnol. 2012, 251364.

Loewe, R.P., Nelson, P.J., 2011. Microarray bioinformatics. Methods Mol. Biol. 671, 295–320.

Logan, C., O'Leary, J.J., O'Sullivan, N., 2006. Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. J. Clin. Microbiol. 44, 3189–95.

Loman, N.J., Constantinidou, C., Chan, J.Z.M., Halachev, M., Sergeant, M., Penn, C.W., Robinson, E.R., Pallen, M.J., 2012. High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. Nat. Rev. Microbiol. 10, 599–606.

Lorenz, I., Earley, B., Gilmore, J., Hogan, I., Kennedy, E., More, S.J., Donovan, G., Dohoo,

I., Montgomery, D., Bennett, F., More, S. 2011. Calf health from birth to weaning. III. housing and management of calf pneumonia. Ir. Vet. J. 64, 14.

Lorenz, T.C., 2012. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. J. Vis. Exp. e3998.

Lorenzetti, E., Alfieri, A.F., Alfieri, A.A., 2016. Isolation of an unusual G26P[13] group A rotavirus strain from piglets with diarrhea in Brazil. Virus Genes 52, 586–91.

Lorenzetti, E., Da Silva Medeiros, T.N., Alfieri, A.F., Alfieri, A.A., 2011. Genetic heterogeneity of wild-type G4P[6] porcine rotavirus strains detected in a diarrhea outbreak in a regularly vaccinated pig herd. Vet. Microbiol. 154, 191–196.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.-H., Wagner, M., 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68, 5064–81.

Lucas, A.S., Swecker, W.S., Lindsay, D.S., Scaglia, G., Neel, J.P.S., Elvinger, F.C., Zajac, A.M., 2014. A study of the level and dynamics of Eimeria populations in naturally infected, grazing beef cattle at various stages of production in the Mid-Atlantic USA. Vet. Parasitol. 202, 201–206.

Luchs, A., Timenetsky, M. do C.S.T., 2014. G8P[6] rotaviruses isolated from Amerindian children in Mato Grosso do Sul, Brazil, during 2009: close relationship of the G and P genes with those of bovine and bat strains. J. Gen. Virol. 95, 627–41.

Ludwig, W., Schleifer, K., Whitman, W., 2009. Revised road map to the phylum Firmicutes. In: De Vos, P., Garrity, G., Jones, D., Krieg, N., Ludwig, W., Rainey, F., Schleifer, K., Whitman, W. (Eds.), The Firmicutes. Springer, New York, NY, pp. 1–14.

Lunardi, M., Headley, S.A., Lisbôa, J.A.N., Amude, A.M., Alfieri, A.A., 2008. Outbreak of acute bovine viral diarrhea in Brazilian beef cattle: Clinicopathological findings and molecular characterization of a wild-type BVDV strain subtype 1b. Res. Vet. Sci. 85, 599–604.

Luo, J., Ge, J., Tang, L., Qiao, X., Jiang, Y., Cui, W., Liu, M., Li, Y., 2012. Development of a loop-mediated isothermal amplification assay for rapid detection of bovine parvovirus. J. Virol. Methods.

Luo, Y., Liang, L., Zhou, L., Zhao, K., Cui, S., 2015. Concurrent infections of pseudorabies virus and porcine bocavirus in China detected by duplex nanoPCR, Journal of Virological Methods.

Lyras, D., O'Connor, J.R., Howarth, P.M., Sambol, S.P., Carter, G.P., Phumoonna, T., Poon, R., Adams, V., Vedantam, G., Johnson, S., Gerding, D.N., Rood, J.I., 2009.

Toxin B is essential for virulence of Clostridium difficile. Nature 458, 1176–9.

Ma, Y., Zhang, Y., Liang, X., Lou, F., Oglesbee, M., Krakowka, S., Li, J. 2015. Origin, evolution, and virulence of Porcine Deltacoronaviruses in the United States. mBio vol. 6 no. 2 e00064-15.

Maclachlan N. J., D.E.J., 2011. Coronaviridae. In: Maclachlan N. J., D.E.J. (Ed.), Fenner's Veterianary Virology. Academic Press Elsevier, San Diego, pp. 393–413.

MacLachlan, N., Dubovi, E., 2011. Reoviridae. In: Fenner's Veterinary Virology. Academic Press, pp. 275–292.

Maddox-Hyttel, C., Langkjær, R.B., Enemark, H.L., Vigre, H., 2006. Cryptosporidium and Giardia in different age groups of Danish cattle and pigs—Occurrence and management associated risk factors. Vet. Parasitol. 141, 48–59.

Mahnel, H., Ottis, K., Herlyn, M., 1977. Stability in drinking and surface water of nine virus species from different genera (author's transl). Zentralbl Bakteriol [Orig B] 164, 64–84.

Malik, Y., Kumar, N., Sharma, K., Sircar, S., Dhama, K., Bora, D., Dutta, T., Prasad, M., Tiwari, A., 2014. Rotavirus diarrhea in piglets: A review on epidemiology, genetic diversity and zoonotic risks. Indian J. Anim. Sci. 84, 1035–1042.

Mallinath, R.H.K., Chikkachowdappa, P.G., Gowda, A.K.J., D'Souza, P.E., 2009. Studies on the prevalence of cryptosporidiosis in bovines in organized dairy farms in and around Bangalore, South India. Vet. Arch. 79, 461–470.

Manber, U., Myers, G., 1993. Suffix arrays: a new method for on-line string searches. Siam J Comput 22, 935–948.

Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. J. Virol. 71, 2562–6.

Mansfield, L.S., Gauthier, D.T., Abner, S.R., Jones, K.M., Wilder, S.R., Urban, J.F., 2003. Enhancement of disease and pathology by synergy of Trichuris suis and Campylobacter jejuni in the colon of immunologically naive swine. Am. J. Trop. Med. Hyg. 68, 70–80.

Mansfield, L.S., Urban, J.F., 1996. The pathogenesis of necrotic proliferative colitis in swine is linked to whipworm induced suppression of mucosal immunity to resident bacteria. Vet. Immunol. Immunopathol. 50, 1–17.

Manteufel, J., Truyen, U., 2009. Animal bocaviruses: A brief review. Intervirology 51, 328–334.

Mao, Z., Zheng, H., Wang, X., Lin, S., Sun, Y., Jiang, B., 2008. DNA microarray for direct identification of bacterial pathogens in human stool samples. Digestion 78, 131–

138.

Markham, N.R., Zuker, M., 2005. DINAMelt web server for nucleic acid melting prediction. Nucleic Acids Res. 33, W577–81.

Markham, N.R., Zuker, M., 2008. UNAFold: software for nucleic acid folding and hybridization. Methods Mol. Biol. 453, 3–31.

Marsteller, T.A., Abvp, D., Armbruster, G., Bane, D.P., Gebhart, C.J., Muller, R., Weatherford, J., Thacker, B., 2003. Monitoring the prevalence of Lawsonia intracellularis IgG antibodies using serial sampling in growing and breeding swine herds. J. Swine Heal. Prod. — 1111, 127127–130.

Martella, V., Bányai, K., Lorusso, E., Bellacicco, A.L., Decaro, N., Mari, V., Saif, L., Costantini, V., De Grazia, S., Pezzotti, G., Lavazza, A., Buonavoglia, C., 2008a. Genetic heterogeneity of porcine enteric caliciviruses identified from diarrhoeic piglets. Virus Genes 36, 365–73.

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C., Ciarlet, M., 2010. Zoonotic aspects of rotaviruses. Vet. Microbiol. 140, 246–55.

Martella, V., Lorusso, E., Banyai, K., Decaro, N., Corrente, M., Elia, G., Cavalli, A., Radogna, A., Costantini, V., Saif, L.J., Lavazza, A., Di Trani, L., Buonavoglia, C., 2008b. Identification of a porcine calicivirus related genetically to human sapoviruses. J Clin Microbiol 46, 1907–1913.

Marthaler, D., Homwong, N., Rossow, K., Culhane, M., Goyal, S., Collins, J., Matthijnssens, J., Ciarlet, M., 2014a. Rapid detection and high occurrence of porcine rotavirus A, B, and C by RT-qPCR in diagnostic samples. J. Virol. Methods 209, 30–34.

Marthaler, D., Rossow, K., Gramer, M., Collins, J., Goyal, S., Tsunemitsu, H., Kuga, K., Suzuki, T., Ciarlet, M., Matthijnssens, J., 2012. Detection of substantial porcine group B rotavirus genetic diversity in the United States, resulting in a modified classification proposal for G genotypes. Virology 433, 85–96.

Marthaler, D., Suzuki, T., Rossow, K., Culhane, M., Collins, J., Goyal, S., Tsunemitsu, H., Ciarlet, M., Matthijnssens, J., 2014b. VP6 genetic diversity, reassortment, intragenic recombination and classification of rotavirus B in American and Japanese pigs. Vet. Microbiol. 172, 359–366.

Martín, M.-J., Martín-Sosa, S., Alonso, J.M., Hueso, P., 2003. Enterotoxigenic Escherichia coli strains bind bovine milk gangliosides in a ceramide-dependent process. Lipids 38, 761–8.

Martínez, M.A., Soto-Del Río, M.D.L.D., Gutiérrez, R.M., Chiu, C.Y., Greninger, A.L., Contreras, J.F., López, S., Arias, C.F., Isa, P., 2015. DNA microarray for detection of gastrointestinal viruses. J. Clin. Microbiol. 53, 136–45.

Matiz, K., Kecskeméti, S., Kiss, I., Adám, Z., Tanyi, J., Nagy, B., 2002. Torovirus detection in faecal specimens of calves and pigs in Hungary: short communication. Acta Vet. Hung. 50, 293–6.

Matsubayashi, M., Kita, T., Narushima, T., Kimata, I., Tani, H., Sasai, K., Baba, E., 2009. Coprological survey of parasitic infections in pigs and cattle in slaughterhouse in Osaka, Japan. J. Vet. Med. Sci. 71, 1079–83.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S.M., Palombo, E.A., Iturriza-Gómara, M., Maes, P., Patton, J.T., Rahman, M., Van Ranst, M., 2008. Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J. Virol. 82, 3204–19.

Matthijnssens, J., Ciarlet, M., McDonald, S.M., Attoui, H., Bányai, K., Brister, J.R., Buesa, J., Esona, M.D., Estes, M.K., Gentsch, J.R., Iturriza-Gómara, M., Johne, R., Kirkwood, C.D., Martella, V., Mertens, P.P.C., Nakagomi, O., Parreño, V., Rahman, M., Ruggeri, F.M., Saif, L.J., Santos, N., Steyer, A., Taniguchi, K., Patton, J.T., Desselberger, U., Van Ranst, M., 2011. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch. Virol. 156, 1397–413.

Matthijnssens, J., Otto, P.H., Ciarlet, M., Desselberger, U., Van Ranst, M., Johne, R., 2012. VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch. Virol. 157, 1177–82.

Maunula, L., Von Bonsdorff, C.-H., 2002. Frequent reassortments may explain the genetic heterogeneity of rotaviruses: analysis of Finnish rotavirus strains. J. Virol. 76, 11793–800.

Maurischat, S., Baumann, B., Martin, A., Malorny, B., 2015. Rapid detection and specific differentiation of Salmonella enterica subsp. enterica Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i:− by real-time multiplex PCR. Int. J. Food Microbiol. 193, 8–14.

Mauroy, A., Scipioni, A., Mathijs, E., Miry, C., Ziant, D., Thys, C., Thiry, E., 2008. Noroviruses and sapoviruses in pigs in Belgium. Arch Virol 153, 1927–1931.

Mauroy, A., Scipioni, A., Mathijs, E., Saegerman, C., Mast, J., Bridger, J.C., Ziant, D., Thys, C., Thiry, E., 2009a. Epidemiological study of bovine norovirus infection by RT-PCR and a VLP-based antibody ELISA. Vet Microbiol 137, 243–251.

Mauroy, A., Scipioni, A., Mathijs, E., Thys, C., Thiry, E., 2009b. Molecular detection of kobuviruses and recombinant noroviruses in cattle in continental Europe. Arch. Virol. 154, 1841–1845.

Maurya, P.S., Rakesh, R.L., Pradeep, B., Kumar, S., Kundu, K., Garg, R., Ram, H., Kumar,

A., Banerjee, P.S., 2013. Prevalence and risk factors associated with Cryptosporidium spp. infection in young domestic livestock in India. Trop. Anim. Health Prod. 45, 941–6.

Mayo, M.A., 2002. A summary of taxonomic changes recently approved by ICTV. Arch. Virol. 147, 1655–63.

McAuliffe, G.N., Anderson, T.P., Stevens, M., Adams, J., Coleman, R., Mahagamasekera, P., Young, S., Henderson, T., Hofmann, M., Jennings, L.C., Murdoch, D.R., 2013. Systematic application of multiplex PCR enhances the detection of bacteria, parasites, and viruses in stool samples. J. Infect. 67, 122–129.

McCluskey, B.J., Haley, C., Rovira, A., Main, R., Zhang, Y., Barder, S., 2016. Retrospective testing and case series study of porcine delta coronavirus in U.S. swine herds. Prev. Vet. Med. 123, 185–191.

McDaniel, T.K., Kaper, J.B., 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. Mol. Microbiol. 23, 399–407.

McDonel, J.L., 1980. Binding of Clostridium perfringens [125I]enterotoxin to rabbit intestinal cells. Biochemistry 19, 4801–4807.

McElhinney, L.M., Cooper, R.J., Morris, D.J., 1995. Multiplex polymerase chain reaction for human herpesvirus-6, human cytomegalovirus, and human beta-globin DNA. J. Virol. Methods 53, 223–33.

McLoughlin, K.S., 2011. Microarrays for pathogen detection and analysis. Brief. Funct. Genomics 10, 342–53.

McNulty, M., Dale, J., Lukert, P., Mankertz, A., Randles, J., Todd, D., 2000. Circoviridae. In: van Regenmortel, C.M.F.M.H.V., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Ed.), Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 299–303.

McOrist, S., Gebhart, C., 1999. Porcine proliferative enteropathies. In: Straw, B., Mengeling, W., D'Allaire, S., Taylor, D. (Eds.), Diseases of Swine. Ames, University Press., Iowa State, USA, pp. 521–534.

McOrist, S., Gebhart, C.., 2006. Proliferative enteropathies. In: Straw, B., Zimmerman, J., D'Allaire, S., Taylor, D. (Eds.), Diseases of Swine. Blackwell Publishing, Oxford, UK, pp. 727–738.

McOrist, S., Jasni, S., Mackie, R.A., MacIntyre, N., Neef, N., Lawson, G.H., 1993. Reproduction of porcine proliferative enteropathy with pure cultures of ileal symbiont intracellularis. Infect. Immun. 61, 4286–92.

Mee J.F., 2008. Newborn dairy calf management. Vet.Clin.North Am. Food Pr. 24, 1–17.

Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. 79 (Pt 9), 2171–9.

Meer, R.R., Songer, J.G., 1997. Multiplex polymerase chain reaction assay for genotyping Clostridium perfringens. Am. J. Vet. Res. 58, 702–5.

Mertens, P., 2004. The dsRNA viruses. Virus Res. 101, 3–13.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J.M., Cornelis, G., 1990. Secretion of Yop proteins by Yersiniae. Infect. Immun. 58, 2840–9.

Midgley, S.E., Bányai, K., Buesa, J., Halaihel, N., Hjulsager, C.K., Jakab, F., Kaplon, J., Larsen, L.E., Monini, M., Poljšak-Prijatelj, M., Pothier, P., Ruggeri, F.M., Steyer, A., Koopmans, M., Böttiger, B., 2012. Diversity and zoonotic potential of rotaviruses in swine and cattle across Europe. Vet. Microbiol. 156, 238–245.

Miller, M.B., Tang, Y.-W.W., 2009. Basic concepts of microarrays and potential applications in clinical microbiology. Clin. Microbiol. Rev. 22, 611–633.

Molinari, B.L.D., Lorenzetti, E., Otonel, R.A.A., Alfieri, A.F., Alfieri, A.A., 2014. Species H rotavirus detected in piglets with diarrhea, Brazil, 2012. Emerg. Infect. Dis. 20, 1019–22.

Molitor, T.W., Joo, H.S., Collett, M.S., 1983. Porcine parvovirus: virus purification and structural and antigenic properties of virion polypeptides. J. Virol. 45, 842–54.

Monis, P.T., Caccio, S.M., Thompson, R.C.A., 2009. Variation in Giardia: towards a taxonomic revision of the genus. Trends Parasitol. 25, 93–100.

Monis, P.T., Giglio, S., 2006. Nucleic acid amplification-based techniques for pathogen detection and identification. Infect. Genet. Evol. 6, 2–12.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J., Megraud, F., 1997. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J. Clin. Microbiol. 35, 995–998.

Moon, H.-J., Song, D., Seon, B.H., Kim, H.-K., Park, S.-J., An, D.-J., Kim, J.-M., Kang, B.-K., Park, B.-K., 2012. Complete Genome Analysis of Porcine Enterovirus B Isolated in Korea. J. Virol. 86, 10250–10250.

Moono, P., Putsathit, P., Knight, D.R., Squire, M.M., Hampson, D.J., Foster, N.F., Riley, T. V., 2016. Persistence of Clostridium difficile RT 237 infection in a Western Australian piggery. Anaerobe 37, 62–66.

Moreno Switt, A.I., Orsi, R.H., den Bakker, H.C., Vongkamjan, K., Altier, C., Wiedmann,

M., 2013. Genomic characterization provides new insight into Salmonella phage diversity. BMC Genomics 14, 481.

Morgan, U., Sargent, K., Deplazes, P., Forbes, D., Spano, F., Hertzberg, H., Elliot, A., Thompson, R., 1998. Molecular characterization of Cryptosporidium from various hosts. Parasitology 117, 31–37.

Mori, Y., and Notomi T, 2009. Loop-mediated isothermal amplification (LAMP): a rapid accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. 15:62-69.

Mothershed, E.A., Whitney, A.M., 2006. Nucleic acid-based methods for the detection of bacterial pathogens: Present and future considerations for the clinical laboratory. Clin. Chim. Acta 363, 206–220.

Moxley, R. a, 2004. Escherichia coli 0157:H7: an update on intestinal colonization and virulence mechanisms. Anim. Health Res. Rev. 5, 15–33.

Moxley, R. a., Smith, D.R., 2010. Attaching-effacing Escherichia coli Infections in Cattle. Vet. Clin. North Am. - Food Anim. Pract. 26, 29–56.

Muir, P., Harbour, D.A., Gruffydd-Jones, T.J., Howard, P.E., Hopper, C.D., Gruffydd-Jones, E.A., Broadhead, H.M., Clarke, C.M., Jones, M.E., 1990. A clinical and microbiological study of cats with protruding nictitating membranes and diarrhoea: isolation of a novel virus. Vet. Rec. 127, 324–30.

Mundt, H.C., Cohnen, A., Daugschies, A., Joachim, A., Prosl, H., Schmaschke, R., Westphal, B., 2005. Occurrence of isospora suis in Germany, Switzerland and Austria. J. Vet. Med. B., 52: 93-97.

Murakami, S., Ogawa, A., Kinoshita, T., Matsumoto, A., Ito, N., Nakane, T., 2006. Occurrence of swine salmonellosis in postweaning multisystemic wasting syndrome (PMWS) affected pigs concurrently infected with porcine reproduction and respiratory syndrome virus (PRRSV). J. Vet. Med. Sci. 68, 387–91.

Myers, L.L., Shoop, D.S., Firehammer, B.D., Border, M.M., 1985. Association of enterotoxigenic Bacteroides fragilis with diarrheal disease in calves. J. Infect. Dis. 152, 1344–7.

Nagai, M., Hayashi, M., Sugita, S., Sakoda, Y., Mori, M., Murakami, T., Ozawa, T., Yamada, N., Akashi, H., 2004. Phylogenetic analysis of bovine viral diarrhea viruses using five different genetic regions. Virus Res 99, 103–113.

Nagy, B., Fekete, P.Z., 1999. Enterotoxigenic Escherichia coli (ETEC) in farm animals. Vet. Res. 30, 259–84.

Nakamura, M., Sato, S., Ohya, T., Suzuki, S., Ikeda, S., 1985. Possible relationship of a 36-megadalton Salmonella enteritidis plasmid to virulence in mice. Infect.

Immun. 47, 831–833.

Nantavisai, K., Mungthin, M., Tan-Ariya, P., Rangsin, R., Naaglor, T., Leelayoova, S., 2007. Evaluation of the sensitivities of DNA extraction and PCR methods for detection of Giardia duodenalis in stool specimens. J. Clin. Microbiol. 45, 581–583.

Nardon, E., Donada, M., Bonin, S., Dotti, I., Stanta, G., 2009. Higher random oligo concentration improves reverse transcription yield of cDNA from bioptic tissues and quantitative RT-PCR reliability. Exp. Mol. Pathol. 87, 146–151.

Naresh, R., Song, Y., Hampson, D.J., 2009. The intestinal spirochete Brachyspira pilosicoli attaches to cultured Caco-2 cells and induces pathological changes. PLoS One 4, e8352.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic Escherichia coli. Clin. Microbiol. Rev. 11, 142–201.

Nathues, H., Oliveira, C.J.B., Wurm, M., Grosse Beilage, E., Givisiez, P.E.N., 2007. Simultaneous Detection of Brachyspira hyodysenteriae, Brachyspira pilosicoli and Lawsonia intracellularis in Porcine Faeces and Tissue Samples by Multiplex-PCR. J. Vet. Med. Ser. A 54, 532–538.

Navarro, E., Serrano-Heras, G., Castaño, M.J.J., Solera, J., 2015. Real-time PCR detection chemistry. Clin. Chim. Acta 439, 231–250.

Ndze, V.N., Cadar, D., Csagola, A., Kisfali, P., Kovacs, E., Farkas, S., Ngu, A.F., Esona, M.D., Dan, A., Tuboly, T., Banyai, K., 2013. Detection of novel porcine bocaviruses in fecal samples of asymptomatic pigs in Cameroon. Infect. Genet. Evol. 17, 277–282.

Ng, J., Yang, R., McCarthy, S., Gordon, C., Hijjawi, N., Ryan, U., 2011. Molecular characterization of Cryptosporidium and Giardia in pre-weaned calves in Western Australia and New South Wales. Vet. Parasitol. 176, 145–150.

Nguyen, S.T., Honma, H., Geurden, T., Ikarash, M., Fukuda, Y., Huynh, V.V., Nguyen, D.T., Nakai, Y., 2012. Prevalence and risk factors associated with Cryptosporidium oocysts shedding in pigs in Central Vietnam. Res. Vet. Sci. 93, 848–852.

Nguyen, T.D., Vo, T.T., Vu-Khac, H., 2011. Virulence factors in Escherichia coli isolated from calves with diarrhea in Vietnam. J. Vet. Sci. 12, 159–164.

Nguyen, T.V., Le Van, P., Le Huy, C., Gia, K.N., Weintraub, A., 2005. Detection and characterization of diarrheagenic Escherichia coli from young children in Hanoi, Vietnam. J. Clin. Microbiol. 43, 755–60.

Ni, J., Qiao, C., Han, X., Han, T., Kang, W., Zi, Z., Cao, Z., Zhai, X., Cai, X., 2014. Identification and genomic characterization of a novel porcine parvovirus (PPV6)

in China. Virol. J. 11, 203.

Nibert, M., Schiff, L., 2001. Reoviruses and their replication. In: Knipe, D., Howley, P. (Eds.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia, pp. 1679–1728.

Niestrath, M., Takla, M., Joachim, A., Daugschies, A., 2002. The role of Isospora suis as a pathogen in conventional piglet production in Germany. J. Vet. Med. B. Infect. Dis. Vet. Public Health 49, 176–80.

Nietfeld, J., 2013. Spiral curved organisms. In: McVey, S., Kennedy, M., Changappa, M. (Eds.), Veterinary Microbiology. Wiley-Blackwell, pp. 167–174.

Nissen, S., Poulsen, I.H., Nejsum, P., Olsen, A., Roepstorff, A., Rubaire-Akiiki, C., Thamsborg, S.M., 2011. Prevalence of gastrointestinal nematodes in growing pigs in Kabale District in Uganda. Trop. Anim. Health Prod. 43, 567–72.

Noel, J.S., Fankhauser, R.L., Ando, T., Monroe, S.S., Glass, R.I., 1999. Identification of a distinct common strain of “Norwalk-like viruses” having a global distribution. J. Infect. Dis. 179, 1334–44.

Norén, T., Johansson, K., Unemo, M., 2014. Clostridium difficile PCR ribotype 046 is common among neonatal pigs and humans in Sweden. Clin. Microbiol. Infect. 20, O2–O6.

Norman, K.N., Harvey, R.B., Scott, H.M., Hume, M.E., Andrews, K., Brawley, A.D., 2009. Varied prevalence of Clostridium difficile in an integrated swine operation. Anaerobe 15, 256–260.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63.

Novotná, M., Škardová, O., 2002. Brachyspira hyodysenteriae: detection, identification and antibiotic susceptibility. Vet. Med. – Czech 47, 104–109.

Nowrousian, M., 2010. Next-generation sequencing techniques for eukaryotic microorganisms: sequencing-based solutions to biological problems. Eukaryot. Cell 9, 1300–10.

Nsofor, C., 2014. DNA microarrays and their applications in medical microbiology. Biotechnol. Mol. Biol. Rev. 9, 1–11.

Nuessen, M.E., Joens, L.A., Glock, R.D., 1983. Involvement of lipopolysaccharide in the pathogenicity of Treponema hyodysenteriae. J. Immunol. 131, 997–9.

O’Connor, A.M., Reed, M.C., Denagamage, T.N., Yoon, K.-J., Sorden, S.D., Cooper, V.L., 2007. Prevalence of calves persistently infected with bovine viral diarrhea virus in beef cow-calf herds enrolled in a voluntary screening project. J. Am. Vet. Med.

Assoc. 230, 1691–6.

O'Handley, R.M., Cockwill, C., McAllister, T.A., Jelinski, M., Morck, D.W., Olson, M.E., 1999. Duration of naturally acquired giardiosis and cryptosporidiosis in dairy calves and their association with diarrhea. J. Am. Vet. Med. Assoc. 214, 391–6.

Oberste, M.S., Maher, K., Pallansch, M.A., 2003. Genomic evidence that simian virus 2 and six other simian picornaviruses represent a new genus in Picornaviridae. Virology 314, 283–293.

Oh, Y., Yang, D., Cho, S., Kang, H., Choi, S., 2011. Sero-surveillance of Transmissible Gastroenteritis Virus (TGEV) and Porcine Respiratory Coronavirus (PRCV) in South Korea 41, 189–193.

Oliver, S.L.L., Asobayire, E., Dastjerdi, A.M.M., Bridger, J.C.C., 2006. Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae. Virology 350, 240–50.

Olson, M.E., O'Handley, R.M., Ralston, B.J., McAllister, T.A., Andrew Thompson, R.., 2004. Update on Cryptosporidium and Giardia infections in cattle. Trends Parasitol. 20, 185–191.

Olson, M.E., Thorlakson, C.L., Deselliers, L., Morck, D.W., McAllister, T.A., 1997. Giardia and Cryptosporidium in Canadian farm animals. Vet. Parasitol. 68, 375–381.

Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357, 175–85.

Oporto, B., Esteban, J.I., Aduriz, G., Juste, R.A., Hurtado, A., 2007. Prevalence and strain diversity of thermophilic campylobacters in cattle, sheep and swine farms. J. Appl. Microbiol. 103, 977–84.

Opriessnig, T., Meng, X.-J., Halbur, P.G., 2007. Porcine Circovirus Type 2 associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J Vet Diagn Invest 19, 591–615.

Otto, P.H., Rosenhain, S., Elschner, M.C., Hotzel, H., Machnowska, P., Trojnar, E., Hoffmann, K., Johne, R., 2015. Detection of rotavirus species A, B and C in domestic mammalian animals with diarrhoea and genotyping of bovine species A rotavirus strains. Vet. Microbiol. 179, 168–176.

Ouardani, M., Wilson, L., Jetté, R., Montpetit, C., Dea, S., 1999. Multiplex PCR for detection and typing of porcine circoviruses. J. Clin. Microbiol. 37, 3917–24.

Ouchene, N., Benakhla, A., Khelifi, N., Righi, S., Paraud, C., Chartier, C., 2012. Prevalence of Cryptosporidium sp in dairy calves in north-eastern Algeria. Méd, Rev. Vét 163, 163–166.

Oxberry, S.L., Hampson, D.J., 2003. Epidemiological studies of Brachyspira pilosicoli in two Australian piggeries. Vet. Microbiol. 93, 109–120.

Pai, C.H., Mors, V., 1978. Production of enterotoxin by Yersinia enterocolitica. Infect. Immun. 19, 908–911.

Pajarillo, E.A.B., Chae, J.P., Marilen, P. B., Hyeun, B.K., Kang-Seok, S., Dae-Kyung, K. 2015. Characterization of the fecal microbial communities of Duroc pigs using 16S rRNA gene pyrosequencing. Asian-Australas J Anim Sci. Apr; 28(4): 584–591.

Pang, X.L., Lee, B., Boroumand, N., Leblanc, B., Preiksaitis, J.K., Yu Ip, C.C., 2004. Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. J. Med. Virol. 72, 496–501.

Pape, J.W., Jean, S.S., Ho, J.L., Hafner, A., Johnson, W.D., 1993. Effect of isoniazid prophylaxis on incidence of active tuberculosis and progression of HIV infection. Lancet 342, 268–272.

Papp, H., László, B., Jakab, F., Ganesh, B., De Grazia, S., Matthijnssens, J., Ciarlet, M., Martella, V., Bányai, K., 2013. Review of group A rotavirus strains reported in swine and cattle. Vet. Microbiol. 165, 190–199.

Paradis, M.A.; Gebhart, C.J.; Toole, D.; Vessie, G.; Winkelman, N.L.; Bauer, S.A.; Wilson, J.B.; McClure, C.A. Sub-clinical ileitis: Diagnostic and perfromance parameters in a multi-dose mucosal homogenate challenge model. J. Swine Health Prod. 20, 137–141.

Parashar, U.D., Gibson, C.J., Bresee, J.S., Glass, R.I., 2006. Rotavirus and severe childhood diarrhea. Emerg. Infect. Dis. 12, 304–6.

Pardon, B., Bleecker, K. De, Hostens, M., Callens, J., Dewulf, J., Deprez, P., 2012. Longitudinal study on morbidity and mortality in white veal calves in Belgium. BMC Vet. Res. 2012 81 8, 420–424.

Park, S-J., Kim, J., Lee, J.S., Rhee, S.K., Kim, H., 2014. Characterization of the fecal microbiome in different swine groups by high-throughput sequencing. Mol. Biol. Genetics and Biotechnol. 28: 157-162.

Park, S., Kim, H., Song, D., Moon, H., Park, B., 2011. Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) field isolates in Korea. Arch. Virol. 156, 577–85.

Park, S.-I.I., Matthijnssens, J., Saif, L.J., Kim, H.-J.J., Park, J.-G.G., Alfajaro, M.M., Kim, D.-S.S., Son, K.-Y.Y., Yang, D.-K.K., Hyun, B.-H.H., Kang, M.-I. Il, Cho, K.-O.O., 2011. Reassortment among bovine, porcine and human rotavirus strains results in G8P[7] and G6P[7] strains isolated from cattle in South Korea. Vet. Microbiol. 152, 55–66.

Park, S.-J., Kim, H.-K., Moon, H.-J., Song, D.-S., Rho, S.-M., Han, J.-Y., Nguyen, V.-G., Park, B.-K., 2010. Molecular detection of porcine kobuviruses in pigs in Korea and their association with diarrhea. Arch. Virol. 155, 1803–11.

Park, S.-J., Kim, H.-K., Song, D.-S., Moon, H.-J., Park, B.-K., 2011. Molecular detection and genetic characterization of kobuviruses in fecal samples collected from diarrheic cattle in Korea. Infect. Genet. Evol. 11, 1178–82.

Park, S.-J.S.-I., Jeong, C., Park, S.-J.S.-I., Kim, H.-H., Jeong, Y.-J., Hyun, B.-H., Chun, Y.-H., Kang, M.-I., Cho, K.-O., 2008. Molecular detection and characterization of unclassified bovine enteric caliciviruses in South Korea. Vet. Microbiol. 130, 371–379.

Park, S.-J.S.-I.S.-H., Jeong, C., Kim, H.-H., Park, S.-J.S.-I.S.-H., Park, S.-J.S.-I.S.-H., Hyun, B.-H., Yang, D.-K., Kim, S.-K., Kang, M.-I., Cho, K.-O., 2007. Molecular epidemiology of bovine noroviruses in South Korea. Vet. Microbiol. 124, 125–133.

Park, S.S.-J., Kim, H.-K.H., Song, D.D.-S., Moon, H.H.-J., Park, B.B.-K., 2011. Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) field isolates in Korea. Arch. Virol. 156, 577–85.

Parker, C.T., Miller, W.G., Horn, S.T., Lastovica, A.J., 2007. Common genomic features of Campylobacter jejuni subsp. doylei strains distinguish them from C. jejuni subsp. jejuni. BMC Microbiol. 7, 50.

Parra, S., Agudelo, T., Ortiz, L., Ramírez, M., Rodríguez, B., Herrera, A., 2011. Lipopolysaccharide (LPS) from E. coli has detrimental effects on the intestinal morphology of weaned pigs. Rev Colomb Cienc Pecu 24, 598–608.

Partridge, S.R., 2015. Resistance mechanisms in Enterobacteriaceae. Pathology 47, 276–284.

Paton, A.W., Paton, J.C., 1998. Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J. Clin. Microbiol. 36, 598–602.

Pearson, G.R., Bazeley, K.J., Jones, J.R., Gunning, R.F., Green, M.J., Cookson, A., Woodward, M.J., 1999. Attaching and effacing lesions in the large intestine of an eight-month-old heifer associated with Escherichia coli O26 infection in a group of animals with dysentery. Vet. Rec. 145, 370–3.

Pearson, G.R., McNulty, M.S., Logan, E.F., 1978. Pathological changes in the small intestine of neonatal calves naturally infected with reo-like virus (rotavirus). Vet. Rec. 102, 454–8.

Pellerin, C., Van Den Hurk, J., Lecomte, J., Tijssen, P., 1994. Identification of a New

Group of Bovine Viral Diarrhea Virus Strains Associated with Severe Outbreaks and High Mortalities. Virology 203, 260–268.

Peña, J.G., 2010. AG-ECO NEWS. Economist-Mgmt 26.

Penrith, M.L., Gerdes, G.H., 1992. Breda virus-like particles in pigs in South Africa. J. S. Afr. Vet. Assoc. 63, 102.

Pensaert, M., Debouck, P., 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch. Virol.

Peplies, J., Glöckner, F.O., Amann, R., 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. 69, 1397–1407.

Perelle, S., Gibert, M., Bourlioux, P., Corthier, G., Popoff, M.R., 1997. Production of a complete binary toxin (actin-specific ADP- ribosyltransferase) by Clostridium difficile CD196. Infect. Immun. 65, 1402–1407.

Pérez, E., Kummeling, A., Janssen, M.M., Jiménez, C., Alvarado, R., Caballero, M., Donado, P., Dwinger, R.H., 1998. Infectious agents associated with diarrhoea of calves in the canton of Tilarán, Costa Rica. Prev. Vet. Med. 33, 195–205.

Persson, S., Torpdahl, M., Olsen, K.E. 2008. New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin. Microbiol. Infect. 14, 1057–64.

Petersen, H.H., Jianmin, W., Katakam, K.K., Mejer, H., Thamsborg, S.M., Dalsgaard, A., Olsen, A., Enemark, H.L., 2015. Cryptosporidium and Giardia in Danish organic pig farms: Seasonal and age-related variation in prevalence, infection intensity and species/genotypes. Vet. Parasitol. 214, 29–39.

Peyret, N., Seneviratne, P.A., Allawi, H.T., SantaLucia, J., 1999. Nearest-neighbor thermodynamics and NMR of DNA sequences with internal A.A, C.C, G.G, and T.T mismatches. Biochemistry 38, 3468–77.

Pham, H.A., Carrique-Mas, J.J., Nguyen, V.C., Ngo, T.H., Nguyet, L.A., Do, T.D., Vo, B.H., Phan, V.T.M., Rabaa, M.A., Farrar, J., Baker, S., Bryant, J.E., 2014. The prevalence and genetic diversity of group A rotaviruses on pig farms in the Mekong Delta region of Vietnam. Vet. Microbiol. 170, 258–65.

Pham, N.T.K., Khamrin, P., Nguyen, T.A., Kanti, D.S., Phan, T.G., Okitsu, S., Ushijima, H., 2007. Isolation and molecular characterization of aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. J. Clin. Microbiol. 45, 2287–2288.

Piao, J., Jiang, J., Xu, B., Wang, X., Guan, Y., Wu, W., Liu, L., Zhang, Y., Huang, X., Wang,

P., Zhao, J., Kang, X., Jiang, H., Cao, Y., Zheng, Y., Jiang, Y., Li, Y., Yang, Y., Chen, W., 2012. Simultaneous Detection and Identification of Enteric Viruses by PCR-Mass Assay. PLoS One 7, e42251.

Picco, N.Y., Alustiza, F.E., Bellingeri, R. V., Grosso, M.C., Motta, C.E., Larriestra, A.J., Vissio, C., Tiranti, K.I., Terzolo, H.R., Moreira, A.R., Vivas, A.B., 2015. Molecular screening of pathogenic Escherichia coli strains isolated from dairy neonatal calves in Cordoba province, Argentina. Rev. Argent. Microbiol. 47, 4–11.

Pignatelli, J., Grau-Roma, L., Jiménez, M., Segalés, J., Rodríguez, D., 2010a. Longitudinal serological and virological study on porcine torovirus (PToV) in piglets from Spanish farms. Vet. Microbiol. 146, 260–8.

Pignatelli, J., Jiménez, M., Grau-Roma, L., Rodríguez, D., 2010b. Detection of porcine torovirus by real time RT-PCR in piglets from a Spanish farm. J. Virol. Methods 163, 398–404.

Pignatelli, J., Jimenez, M., Luque, J., Rejas, M.T., Lavazza, a, Rodriguez, D., 2009. Molecular characterization of a new PToV strain. Evolutionary implications. Virus Res. 143, 33–43.

Pinheiro de Oliveira, T.F., Fonseca Júnior, A.A., Camargos, M.F., de Oliveira, A.M., Lima, N.F., Freitas, M.E., de Oliveira Guedes, E., de Azevedo, I.C., Pinto Cottorello, A.C., Heinemann, M.B., 2016. Porcine parvovirus as a contaminant in cell cultures and laboratory supplies. Biologicals 44, 53–59.

Platt-Samoraj, A., Ugorski, M., Szweda, W., Szczerba-Turek, A., Wojciech, K., Procajło, Z., 2006. Analysis of the presence of ail, ystA and ystB genes in Yersinia enterocolitica strains isolated from aborting sows and aborted fetuses. J. Vet. Med. Ser. B Infect. Dis. Vet. Public Heal. 53, 341–346.

Platts-Mills, J. a, Operario, D.J., Houpt, E.R., 2012. Molecular diagnosis of diarrhea: current status and future potential. Curr. Infect. Dis. Rep. 14, 41–6.

Pogranichniy, R.M., Janke, B.H., Gillespie, T.G., Yoon, K.-J., 2003. A prolonged outbreak of polioencephalomyelitis due to infection with a group I porcine enterovirus. J. Vet. Diagn. Invest. 15, 191–4.

Polak, M.P., Kuta, A., Rybaltowski, W., Rola, J., Larska, M., Zmudzinski, J.F., 2014. First report of bovine viral diarrhoea virus-2 infection in cattle in Poland. Vet. J. 202, 643–645.

Poljak, Z., Dewey, C.E., Martin, S.W., Rosendal, T., Christensen, J., Ciebin, B., Friendship, R.M., 2010. Prevalence of Yersinia enterocolitica shedding and bioserotype distribution in Ontario finisher pig herds in 2001, 2002, and 2004. Prev. Vet. Med. 93, 110–20.

Popoff, M.R., 2011. Multifaceted Interactions of Bacterial Toxins With the Gastrointestinal Mucosa. Future Microbiol. 6(7): 763-797.

Poulsen, L., Søe, M.J., Snakenborg, D., Møller, L.B., Dufva, M., 2008. Multi-stringency wash of partially hybridized 60-mer probes reveals that the stringency along the probe decreases with distance from the microarray surface. Nucleic Acids Res. 36, e132.

Pourtaghi, H., Dahpahlavan, V., Momtaz, H., 2013. Virulence genes in Escherichia coli isolated from calves with diarrhoea in Iran. Comp. Clin. Path. 22, 513–515.

Procop, G.W., 2007. Molecular diagnostics for the detection and characterization of microbial pathogens. Clin. Infect. Dis. 45 Suppl 2, S99–S111.

Prodělalová, J., 2012. The survey of porcine teschoviruses, sapeloviruses and enteroviruses B infecting domestic pigs and wild boars in the Czech Republic between 2005 and 2011. Infect. Genet. Evol. 12, 1447–1451.

Puranaveja, S., Poolperm, P., Lertwatcharasarakul, P., Kesdaengsakonwut, S., Boonsoongnern, A., Urairong, K., Kitikoon, P., Choojai, P., Kedkovid, R., Teankum, K., Thanawongnuwech, R., 2009. Chinese-like strain of porcine epidemic diarrhea virus, Thailand. Emerg. Infect. Dis. 15, 1112–5.

Qi, M.Z., Fang, Y.Q., Wang, X.T., Zhang, L.X., Wang, R.J., Du, S.Z., Guo, Y.X., Jia, Y.Q., Yao, L., Liu, Q.D., Zhao, G.H., 2015. Molecular characterization of Cryptosporidium spp. in pre-weaned calves in Shaanxi Province, north-western China. J. Med. Microbiol. 64, 111–6.

Qu, W., Zhou, Y., Zhang, Y., Lu, Y., Wang, X., Zhao, D., Yang, Y., Zhang, C., 2012. MFEprimer-2.0: a fast thermodynamics-based program for checking PCR primer specificity. Nucleic Acids Res. 40, W205–8.

Quinn, P., Markey, B., Leonard, F., Fitzpatrick, E., Fanning, S., Hartigan, P., 2011. Reovirideae family. In: Quinn, P., Markey, B., Leonard, F., Fitzpatrick, E., Fanning, S., Hartigan, P. (Eds.), Veterinary Microbiology and Microbial Disease. John Wiley & Sons, pp. 635–643.

Racaniello, V.R., 2007. Picornaviridae: the viruses and their replication. In: Knipe, DM, Howley, P. (Ed.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 795–838.

Radwan, G.S., Brock, K. V., Hogan, J.S., Larry Smith, K., 1995. Development of a PCR amplification assay as a screening test using bulk milk samples for identifying dairy herds infected with bovine viral diarrhea virus. Vet. Microbiol. 44, 77–91.

Rahman, M., Banik, S., Faruque, A.S.G., Taniguchi, K., Sack, D.A., Van Ranst, M., Azim, T., 2005. Detection and characterization of human group C rotaviruses in Bangladesh. J. Clin. Microbiol. 43, 4460–5.

Ranz, A.I., Manclus, J.J., Diaz-Aroca, E., Casal, J.I., 1989. Porcine Parvovirus: DNA Sequence and Genome Organization. J. Gen. Virol. 70, 2541–2553.

Reller L.B., Weinstein, M. P., Petti, C. A., 2007. Detection and Identification of Microorganisms by Gene Amplification and Sequencing. Clin. Infec. Dis. 44: 1108-1114.

Relógio, A., Schwager, C., Richter, A., Ansorge, W., Valcárcel, J., 2002. Optimization of oligonucleotide-based DNA microarrays. Nucleic Acids Res. 30, e51.

Ren, L., Zhang, W., Yang, S., Shen, Q., Fan, K., Hua, X., 2012. Sequencing of a porcine enterovirus strain prevalent in swine groups in China and recombination analysis. Vet. Microbiol. 159, 265–268.

Ren, X., Li, P., 2011. Development of reverse transcription loop-mediated isothermal amplification for rapid detection of porcine epidemic diarrhea virus. Virus Genes 42, 229–35.

Reuter, G., Boldizsár, A., Kiss, I., Pankovics, P., 2008. Candidate new species of Kobuvirus in porcine hosts. Emerg. Infect. Dis. 14, 1968–70.

Reuter, G., Boldizsár, Á., Pankovics, P., Boldizsár, A., Pankovics, P., 2009. Complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus, a member of a new species in the genus Kobuvirus, family Picornaviridae. Arch. Virol. 154, 101–108.

Reuter, G., Egyed, L., 2009. Bovine kobuvirus in Europe. Emerg. Infect. Dis. 15, 822–3.

Reuter, G., Kecskémeti, S., Pankovics, P., 2010a. Evolution of porcine kobuvirus infection, Hungary. Emerg. Infect. Dis. 16, 696–8.

Reuter, G., Pankovics, P., Knowles, N.J., Boros, Á., 2012. Two closely related novel picornaviruses in cattle and sheep in Hungary from 2008 to 2009, proposed as members of a new genus in the family Picornaviridae. J. Virol. 86, 13295–302.

Reuter, G., Zimsek-Mijovski, J., Poljsak-Prijatelj, M., Di Bartolo, I., Ruggeri, F.M., Kantala, T., Maunula, L., Kiss, I., Kecskeméti, S., Halaihel, N., Buesa, J., Johnsen, C., Hjulsager, C.K., Larsen, L.E., Koopmans, M., Böttiger, B., 2010b. Incidence, diversity, and molecular epidemiology of sapoviruses in swine across Europe. J. Clin. Microbiol. 48, 363–8.

Reynolds, D.J., Morgan, J.H., Chanter, N., Jones, P.W., Bridger, J.C., Debney, T.G., Bunch, K.J., 1986. Microbiology of calf diarrhoea in southern Britain. Vet. Rec. 119, 34–9.

Ribeiro, V.B., Lincopan, N., Landgraf, M., Franco, B.D.G.M., Destro, M.T., 2011. Characterization of class 1 integrons and antibiotic resistance genes in multidrug-

resistant Salmonella enterica isolates from foodstuff and related sources. Braz. J. Microbiol. 42, 685–92.

Riccelli, P. V, Merante, F., Leung, K.T., Bortolin, S., Zastawny, R.L., Janeczko, R., Benight, A.S., 2001. Hybridization of single-stranded DNA targets to immobilized complementary DNA probes : comparison of hairpin versus linear capture probes. Nucleic Acids Res. 29, 996–1004.

Richter, B., Ladinig, A., Nedorost, N., Weissenböck, H., 2010. A TaqMan quantitative polymerase chain reaction assay for the detection of Lawsonia intracellularis in fecal and tissue samples from pigs. J. Vet. Diagn. Invest. 22, 70–3.

Ridpath, J.F., 2010. Bovine Viral Diarrhea Virus: Global Status. Vet. Clin. North Am. - Food Anim. Pract. 26, 105–121.

Ridpath, J.F., Bolin, S.R., Dubovi, E.J., 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205, 66–74.

Ridpath, J.F., Neill, J.D., Vilcek, S., Dubovi, E.J., Carman, S., 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Vet. Microbiol. 114, 196–204.

Riley, D.R., Sieber, K.B., Robinson, K.M., White, J.R., Ganesan, A., Nourbakhsh, S., Dunning Hotopp J.C. 2013. Bacteria-human somatic cell lateral gene transfer is enriched in cancer samples. PLoS Comput. Biol., 9 (6) (2013), p. e1003107.

Rimour, S., Hill, D., Militon, C., Peyret, P., 2005. GoArray : highly dynamic and efficient microarray probe design. Bioinformatics. 21, 1094–1103.

Rizal, A., Kumar, A., Vidyarthi, A.S., 2010. Prevalence of Pathogenic Genes in Campylobacter jejuni Isolated from Poultry and Human. Internet J. Food Saf. 12, 29–34.

Rochelle, P.A., De Leon, R., Stewart, M.H., Wolfe, R.L., 1997. Comparison of primers and optimization of PCR conditions for detection of Cryptosporidium parvum and Giardia lamblia in water. Appl. Environ. Microbiol. 63, 106–14.

Rodriguez-Palacios, A., Stämpfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., Weese, J.S., 2006. Clostridium difficile PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12, 1730–1736.

ROSEN, L., 1962. Reoviruses in animals other than man. Ann. N. Y. Acad. Sci. 101, 461–5.

Rotger, R., Casadesús, J., 1999. The virulence plasmids of Salmonella. Int. Microbiol. 2, 177–84.

Rouse, R.J.D., Field, K., Lapira, J., Lee, A., Wick, I., Eckhardt, C., Bhasker, C.R., Soverchia, L., Hardiman, G., 2008. Development and application of a microarray meter tool

to optimize microarray experiments. BMC Res. Notes 1, 45.

Ruckdeschel, K., Roggenkamp, A., Schubert, S., Heesemann, J., 1996. Differential contribution of Yersinia enterocolitica virulence factors to evasion of microbicidal action of neutrophils. Infect. Immun. 64, 724–733.

Rudi, K., Nogva, H.K., Naterstad, K., Drømtorp, S.M., Bredholt, S., Holck, A., 2003. Subtyping Listeria monocytogenes through the combined analyses of genotype and expression of the hlyA virulence determinant. J. Appl. Microbiol. 94, 720–32.

Ruest, N., Couture, Y., Faubert, G.M., Girard, C., 1997. Morphological changes in the jejunum of calves naturally infected with Giardia spp. and Cryptosporidium spp. Vet. Parasitol. 69, 177–186.

Ruiz, V.L.A., Bersano, J.G., Carvalho, A.F., Catroxo, M.H.B., Chiebao, D.P., Gregori, F., Miyashiro, S., Nassar, A.F.C., Oliveira, T.M.F.S., Ogata, R.A., Scarcelli, E.P., Tonietti, P.O., 2016. Case-control study of pathogens involved in piglet diarrhea. BMC Res. Notes 9, 22.

Rutter, J.M., Beer, R.J., 1975. Synergism between Trichuris suis and the microbial flora of the large intestine causing dysentery in pigs. Infect. Immun. 11, 395–404.

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C.A., Xiao, L., 2004. Cryptosporidium suis n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (Sus scrofa). J. Parasitol. 90, 769–73.

SABIN, A.B., 1959. Reoviruses. A new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. Science 130, 1387–9.

Saif, L.J. and Wesley, R.D., 1992. Transmissible Gastroenteritis. In: Leman, AD , Strauss, BE, Mengeling WL, D'Allaire, S, T.D. (Ed.), Diseases of Swine. Iowa State University Press, Iowa State, pp. 362–386.

Saif, L.J. and Wesley, R.D., 1999. Transmissible gastroenteritis and porcine respiratory coronavirus. In: Straw, B. E., D'Allaire, S., Mengeling, W. L., Taylor, D.I. (Ed.), Diseases of Swine. Ames, University Press., Iowa State, pp. 295–325.

Saif, L., Rosen, B., Parwani, A., 1994. Animal Rotaviruses. In: Kapikian, A. (Ed.), Viral Infections of the Gastrointestinal Tract. Marcel Dekker, Inc, New York, NY, pp. 279–367.

Saif, L.J., 1990. A review of evidence implicating bovine coronavirus in the etiology of winter dysentery in cows: an enigma resolved? Cornell Vet. 80, 303–11.

Saif, L.J., Bohl, E.H., Theil, K.W., Cross, R.F., House, J.A., 1980. Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. J. Clin. Microbiol. 12, 105–11.

Saif, L.J., Jiang, B., 1994. Nongroup A rotaviruses of humans and animals. Curr. Top. Microbiol. Immunol. 185, 339–71.

Salles, M.W.S., Scholes, S.F.E., Dauber, M., Strebelow, G., Wojnarowicz, C., Hassard, L., Acton, A.C., Bollinger, T.K., 2011. Porcine teschovirus polioencephalomyelitis in western Canada. J. Vet. Diagn. Invest. 23, 367–373.

Sánchez-Fauquier, A., Roman, E., Colomina, J., Wilhelmi, I., Glass, R.I., Jiang, B., 2003. First detection of group C rotavirus in children with acute diarrhea in Spain. Arch. Virol. 148, 399–404.

Sandals, W.C., Povey, R.C., Meek, A.H., 1995. Prevalence of bovine parvovirus infection in Ontario dairy cattle. Can. J. Vet. Res. 59, 81–86.

Sandhu, K.S., Gyles, C.L., 2002. Pathogenic Shiga toxin-producing Escherichia coli in the intestine of calves. Can. J. Vet. Res. 66, 65–72.

Sanger, F., Coulson, A.R., 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. 94, 441–8.

SantaLucia, J., 1998. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc. Natl. Acad. Sci. U. S. A. 95, 1460–5.

SantaLucia, J., Hicks, D., 2004. The thermodynamics of DNA structural motifs. Annu. Rev. Biophys. Biomol. Struct. 33, 415–40.

Santín, M., Trout, J.M., Xiao, L., Zhou, L., Greiner, E., Fayer, R., 2004. Prevalence and age-related variation of Cryptosporidium species and genotypes in dairy calves. Vet. Parasitol. 122, 103–17.

Sarkar, G., Pelletier, J., Bassel-Duby, R., Jayasuriya, A., Fields, B.N., Sonenberg, N., 1985. Identification of a new polypeptide coded by Identification of a New Polypeptide Coded by Reovirus Gene S1. J. Virol. 54, 720–725.

Sarker, M.R., Carman, R.J., McClane, B.A., 1999. Inactivation of the gene (cpe) encoding Clostridium perfringens enterotoxin eliminates the ability of two cpe-positive C. perfringens type A human gastrointestinal disease isolates to affect rabbit ileal loops. Mol. Microbiol. 33, 946–958.

Sartor, M., Schwanekamp, J., Halbleib, D., Mohamed, I., Karyala, S., Medvedovic, M., Tomlinson, C.R., 2004. Microarray results improve significantly as hybridization approaches equilibrium. Biotechniques 36, 790–796.

Sasaki, J., Nagashima, S., Taniguchi, K., 2003. Aichi Virus Leader Protein Is Involved in Viral RNA Replication and Encapsidation. J. Virol. 77, 10799–10807.

Saunders, N.A., 2004. Real-time PCR. Methods Mol. Biol. 266, 191–211.

Savarino, S.J., McVeigh, A., Watson, J., Cravioto, A., Molina, J., Echeverria, P., Bhan, M.K., Levine, M.M., Fasano, A., 1996. Enteroaggregative Escherichia coli heat-

stable enterotoxin is not restricted to enteroaggregative E. coli. J. Infect. Dis. 173, 1019–22.

Sayeed, S., Li, J., McClane, B.A., 2010. Characterization of virulence plasmid diversity among Clostridium perfringens type B isolates. Infect. Immun. 78, 495–504.

Schauer, D.B., McCathey, S.N., Daft, B.M., Jha, S.S., Tatterson, L.E., Taylor, N.S., Fox, J.G., 1998. Proliferative enterocolitis associated with dual infection with enteropathogenic Escherichia coli and Lawsonia intracellularis in rabbits. J. Clin. Microbiol. 36, 1700–3.

Schena, M., Shalon, D., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467–70.

Scherer, C., Sperhacke, R.D., Ruffino-Netto, A., Rossetti, M.L.R., Vater, C., Klatser, P., Kritski, A.L. 2009. Cost-effectiveness analysis of PCR for the rapid diagnosis of pulmonary tuberculosis. BMC Inf. Dis. 20099:216.

Scherr, M., Rossi, J.J., Sczakiel, G., Patzel, V., 2000. RNA accessibility prediction: a theoretical approach is consistent with experimental studies in cell extracts. Nucleic Acids Res. 28, 2455–61.

Schiff, L., Nibert, M., Tyler, K., 2007. Orthoreoviruses and their replication. In: Knipe, D., Griffin, D., Lamb, R., Straus, S., Howley, P., Martin, M., Roizman, B. (Eds.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia, pp. 1853–1915.

Schirrmeier, H., 2014. Three years of mandatory BVD control in Germany - Lessons to be learned. In: Proceedings XXVIII World Buiatrics Congress, Cairns 2014. Caims.

Schmoock, G., Ehricht, R., Sprague, L.D., 2014. DNA microarray-based detection of Coxiella burnetii, the causative agent of Q fever. Acta Vet. Scand. 56, 27.

Schrenzel, J., Kostic, T., Bodrossy, L., Francois, P., 2009. Introduction to Microarray-Based Detection Methods. In: Kostic, T., Butaye, P., Schrenzel, J. (Eds.), Detection of Highly Dangerous Pathogens: Microarray Methods for the Detection of BSL 3 and BSL 4 Agents. WILEY-VCH Verlag GmbH & Co, KGaA, Weinhei, Germany, pp. 1–34.

Schroeder, M.E., Bounpheng, M. a, Rodgers, S., Baker, R.J., Black, W., Naikare, H., Velayudhan, B., Sneed, L., Szonyi, B., Clavijo, A., 2012. Development and performance evaluation of calf diarrhea pathogen nucleic acid purification and detection workflow. J. Vet. Diagn. Invest. 24, 945–53.

Schuffenecker, I., Ando, T., Thouvenot, D., Lina, B., Aymard, M., 2001. Genetic classification of “Sapporo-like viruses”;. Arch. Virol. 146, 2115–32.

Schulze, F., 1992. [Campylobacter as the cause of diarrhea in calves]. DTW. Dtsch.

tierärztliche Wochenschrift 99, 458–61.

Scipioni, A., Mauroy, A., Vinjé, J., Thiry, E., 2008. Animal noroviruses. Vet. J. 178, 32–45.

Scott, A.C., Chaplin, M.J., Stack, M.J., Lund, L.J., 1987. Porcine torovirus? Vet. Rec. 120, 583.

Sears, C.L., 2009. Enterotoxigenic Bacteroides fragilis: a rogue among symbiotes. Clin. Microbiol. Rev. 22, 349–69, Table of Contents.

Seelenfreund, E., Robinson, W.A., Amato, C.M., Tan, A.-C., Kim, J., Robinson, S.E. 2014. Long Term Storage of Dry versus Frozen RNA for Next Generation Molecular Studies. PLoS One 9, e111827.

Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grassland, B., Baekbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. Vet. Rec. 162, 867–8.

Selwet, M., Galbas, M., 2012. Monitoring selected genes in Campylobacter jejuni and Campylobacter coli isolates from domestic animals. Bull Vet Inst Pulawy 56, 283–286.

Seppä-Lassila, L., Orro, T., Lassen, B., Lasonen, R., Autio, T., Pelkonen, S., Soveri, T., 2015. Intestinal pathogens, diarrhoea and acute phase proteins in naturally infected dairy calves. Comp. Immunol. Microbiol. Infect. Dis. 41, 10–16.

Shahrani, M., Dehkordi, F.S., Momtaz, H., 2014. Characterization of Escherichia coli virulence genes, pathotypes and antibiotic resistance properties in diarrheic calves in Iran. Biol. Res. 47, 28.

Shalon, D., Smith, S.J., Brown, P.O., 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res. 6, 639–45.

Shams, Z., Tahamtan, Y., Pourbakhsh, A., Hosseiny, M.H., Kargar, M., Hayati, M., 2012. Detection of enterotoxigenic K99 (F5) and F41 from fecal sample of calves by molecular and serological methods. Comp. Clin. Path. 21, 475–478.

Shan, T., Lan, D., Li, L., Wang, C., Cui, L., Zhang, W., Hua, X., Zhu, C., Zhao, W., Delwart, E., 2011. Genomic characterization and high prevalence of bocaviruses in swine. PLoS One 6, e17292.

Shankar, E.M., Vignesh, R., Ellegård, R., Barathan, M., Chong, Y.K., Bador, M.K., Rukumani, D. V, Sabet, N.S., Kamarulzaman, A., Velu, V., Larsson, M. 2014. HIV-Mycobacterium tuberculosis co-infection: a “danger-couple model” of disease

pathogenesis. Pathog. Dis. 70, 110–8.

Shatkin, A.J., Sipe, J.D., Loh, P., 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. 2, 986–91.

Shaukat, S., Angez, M., Alam, M.M., Sharif, S., Khurshid, A., Malik, F., Rana, M.S., Mahmood, T., Zaidi, S.S.Z., 2012. Molecular identification and characterization of a new type of bovine enterovirus. Appl. Environ. Microbiol. 78, 4497–500.

Shchepinov, M.S., Case-Green, S.C., Southern, E.M., 1997. Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. Nucleic Acids Res. 25, 1155–61.

Sheather, L., 1923. The detection of intestinal protozoa and mange parasites by a flotation technique. J. Comp. Path. Ther. 36, 266–275.

Shetab, R., Cohen, S.H., Prindiville, T., Tang, Y.J., Cantrell, M., Rahmani, D., Silva, J., 1998. Detection of Bacteroides fragilis Enterotoxin Gene by PCR. J. Clin. Microbiol. 36, 1729–1732.

Shimizu, R., Osawa, K., Shigemura, K., Yoshida, H., Fujiwara, M., Iijima, Y., Fujisawa, M., Shirakawa, T., 2014. Development of multiplex pcr for rapid identification of four salmonella serovars most commonly isolated in Japan. SouthEast Asian J trop Med public Heal. 654.

Shinde, S.B., Fernandes, C.B., Patravale, V.B., 2012. Recent trends in in-vitro nanodiagnostics for detection of pathogens. J. Control. Release 159, 164–180.

Sibley, C.D., Peirano, G., Church, D.L., 2012. Molecular methods for pathogen and microbial community detection and characterization: Current and potential application in diagnostic microbiology. Infect. Genet. Evol. 12, 505–521.

Siegmund, K.H., Steiner, U.E., Richert, C., 2003. ChipCheck--a program predicting total hybridization equilibria for DNA binding to small oligonucleotide microarrays. J. Chem. Inf. Comput. Sci. 43, 2153–62.

Simmonds, P., 2006. Recombination and selection in the evolution of picornaviruses and other Mammalian positive-stranded RNA viruses. J. Virol. 80, 11124–11140.

Simonová, J., Vázlerová, M., Steinhauserová, I., 2007. Detection of Pathogenic Yersinia enterocolitica Serotype O:3 by Biochemical, Serological, and PCR Methods. Czech J. Food Sci 214, 214–220.

Simpson, A.A., Hébert, B., Sullivan, G.M., Parrish, C.R., Zádori, Z., Tijssen, P., Rossmann, M.G., 2002. The structure of porcine parvovirus: comparison with related viruses. J. Mol. Biol. 315, 1189–1198.

Singh, R., Nitsche, J., Andreadis, S.T., 2009. An integrated reaction-transport model for DNA surface hybridization: Implications for DNA microarrays. Ann. Biomed. Eng.

37, 255–269.

Sinha, A., Gauger, P., Zhang, J., Yoon, K.-J., Harmon, K., 2015. PCR-based retrospective evaluation of diagnostic samples for emergence of porcine deltacoronavirus in US swine, Veterinary Microbiology.

Sjöling, Å., Sadeghipoorjahromi, L., Novak, D., Tobias, J., 2015. Detection of major diarrheagenic bacterial pathogens by multiplex PCR panels. Microbiol. Res. 172, 34–40.

Skurnik, M., Venho, R., Bengoechea, J.A., Moriyón, I., 1999. The lipopolysaccharide outer core of Yersinia enterocolitica serotype O:3 is required for virulence and plays a role in outer membrane integrity. Mol. Microbiol. 31, 1443–62.

Smíd, B., Valícek, L., Rodák, L., Kudrna, J., Musilová, J., 1993. [Detection of porcine epidemic diarrhea virus using electron microscopy in the Czech Republic]. Veterinární medicína 38, 333–41.

Smiley, J.R., Chang, K.O., Hayes, J., Vinjé, J., Saif, L.J., 2002. Characterization of an enteropathogenic bovine calicivirus representing a potentially new calicivirus genus. J. Virol. 76, 10089–98.

Smiley, J.R., Hoet, A.E., Tråvén, M., Tsunemitsu, H., Saif, L.J., 2003. Reverse transcription-PCR assays for detection of bovine enteric caliciviruses (BEC) and analysis of the genetic relationships among BEC and human caliciviruses. J. Clin. Microbiol. 41, 3089–99.

Smitalova, R. ,Vyzkumn. U.V.L.B. (Czech R., Rodak, L. ,Vyzkumn. U.V.L.B. (Czech R., Smid, B. ,Vyzkumn. U.V.L.B. (Czech R., Psikal, I. ,Vyzkumn. U.V.L.B. (Czech R., 2009. Detection of nongroup A rotaviruses in faecal samples of pigs in the Czech Republic. Vet. Med. - UZEI (Czech Republic).

Smith, G.W., Berchtold, J., 2014. Fluid Therapy in Calves. Vet. Clin. North Am. Food Anim. Pract. 30, 409–427.

Smith, K., Diggle, M.A., Clarke, S.C., 2004. Automation of a fluorescence-based multiplex PCR for the laboratory confirmation of common bacterial pathogens. J. Med. Microbiol. 53, 115–7.

Smits, S.L., Lavazza, A., Matiz, K., Horzinek, M.C., Koopmans, M.P., de Groot, R.J., 2003. Phylogenetic and evolutionary relationships among torovirus field variants: evidence for multiple intertypic recombination events. J. Virol. 77, 9567–77.

Snijder, E.J., Horzinek, M.C., 1993. Review article Toroviruses : replication, evolution and comparison with other members of the coronavirus-like superfamily 2305–2316.

Snodgrass, D.R., Terzolo, H.R., Sherwood, D., Campbell, I., Menzies, J.D., Synge, B.A.,

1986. Aetiology of diarrhoea in young calves. Vet. Rec. 119, 31–4.

Song, D., Moon, H., Kang, B., 2015. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. Clin. Exp. Vaccine Res. 4, 166–76.

Songer, J., Glock, R., 1998. Enteric infection of swine with Clostridium perfringens types A and C. Swine Heal. Prod. — 6, 223–225.

Songer, J.G., 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9, 216–34.

Songer, J.G., Post, K.W., Larson, D.J., Jost, B.H., Glock, R.D., 2000. Infection of neonatal swine with Clostridium difficile. Swine Heal. Prod. 8, 185–189.

Songer, J.G., Uzal, F. a., 2005. Clostridial Enteric Infections in Pigs. J. Vet. Diagnostic Investig. 17, 528–536.

Sosnovtsev, S. V, Belliot, G., Chang, K.-O., Prikhodko, V.G., Thackray, L.B., Wobus, C.E., Karst, S.M., Virgin, H.W., Green, K.Y., 2006. Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. J. Virol. 80, 7816–31.

Sozzi, E., Barbieri, I., Lavazza, A, Lelli, D., Moreno, a, Canelli, E., Bugnetti, M., Cordioli, P., 2010. Molecular characterization and phylogenetic analysis of VP1 of porcine enteric picornaviruses isolates in Italy. Transbound. Emerg. Dis. 57, 434–42.

Squire, M.M., Carter, G.P., Mackin, K.E., Chakravorty, A., Norén, T., Elliott, B., Lyras, D., Riley, T. V., 2013. Novel molecular type of clostridium difficile in neonatal pigs, Western Australia. Emerg. Infect. Dis. 19, 790–792.

Stackebrandt, E., Goebel, B.M., 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. Int. J. Syst. Bacteriol. 44, 846–849.

Ståhl, K., Alenius, S., 2012. BVDV control and eradication in Europe —an update. Jpn. J. Vet. Res. 60, 31–39.

Ståhlberg, A., Håkansson, J., Xian, X., Semb, H., Kubista, M., 2004. Properties of the Reverse Transcription Reaction in mRNA Quantification. Clin. Chem. 50, 509–515.

Stanley, N.F., 1967. Reoviruses. Br. Med. Bull. 23, 150–4.

Stanton, T.B., Rosey, E.L., Kennedy, M.J., Jensen, N.S., Bosworth, B.T., 1999. Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete Brachyspira (Serpulina) hyodysenteriae, etiologic agent of swine dysentery. Appl. Environ. Microbiol. 65, 5028–34.

Stappers, M.H.T., Hagen, F., Reimnitz, P., Mouton, J.W., Meis, J.F., Gyssens, I.C. 2016. Bacteroides fragilis in biopsies of patients with major abscesses and diabetic foot

infections: direct molecular versus culture-based detection. Diagn. Microbiol. Infect. Dis. 85, 263–265.

Steele, J., Feng, H., Parry, N., Tzipori, S., 2010. Piglet models of acute or chronic Clostridium difficile illness. J. Infect. Dis. 201, 428–34.

Stege, H., Jensen, T.K.K., Møller, K., Vestergaard, K., Baekbo, P., Jorsal, S.E.E., 2004. Infection dynamics of Lawsonia intracellularis in pig herds. Vet. Microbiol. 104, 197–206.

Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J. Vet. Diagn. Invest. 25, 649–54.

Stevenson, H.S., Wang, Y., Muller, R., Edelman, D.C., 2015. Long-term Stability of Total RNA in RNAstable as Evaluated by Expression Microarray. Biopreserv.Biobank. 13, 114–122.

Stewart, F.J., Cavanaugh, C.M., 2007. Intragenomic variation and evolution of the internal transcribed spacer of the rRNA operon in bacteria. J. Mol. Evol. 65, 44–67.

Stockdale, P.H., Bainborough, A.R., Bailey, C.B., Niilo, L., 1981. Some pathophysiological changes associated with infection of Eimeria zuernii in calves. Can. J. Comp. Med. Rev. Can. médecine Comp. 45, 34–7.

Stone, G.G., Oberst, R.D., Hays, M.P., McVey, S., Chengappa, M.M., 1994. Detection of Salmonella serovars from clinical samples by enrichment broth cultivation-PCR procedure. J. Clin. Microbiol. 32, 1742–9.

Storz, J., 1998. Respiratory disease of cattle associated with coronavirus infections. In: Howard, JL, Smith, R. (Ed.), Current Veterinary Therapy: Food Animal Practice. WB Saunders, Philadelphia, pp. 291–293.

Storz, J., Stine, L., Liem, A., Anderson, G.A., 1996. Coronavirus isolation from nasal swab samples in cattle with signs of respiratory tract disease after shipping. J. Am. Vet. Med. Assoc. 208, 1452–5.

Straus, N.A., Bonner, T.I., 1972. Temperature dependence of RNA-DNA hybridization kinetics. BBA Sect. Nucleic Acids Protein Synth. 277, 87–95.

Streck, A.F., Homeier, T., Foerster, T., Fischer, S., Truyen, U., 2013. Analysis of porcine parvoviruses in tonsils and hearts from healthy pigs reveals high prevalence and genetic diversity in Germany. Arch. Virol. 158, 1173–1180.

Stucki, U., Frey, J., Nicolet, J., Burnens, A.P., 1995. Identification of Campylobacter

jejuni on the basis of a species-specific gene that encodes a membrane protein. J. Clin. Microbiol. 33, 855–9.

Suh, D.K., Song, J.C., 2005. Prevalence of Lawsonia intracellularis, Brachyspira hyodysenteriae and Salmonella in swine herds. J. Vet. Sci. 6, 289–93.

Sun, H., Mo, Q.-H., Lin, J.-C., Yang, Z., Tu, C.-N., Gu, D.-Y., Shi, L., Lu, W.-P., 2010. Rapid simultaneous screening of seven clinically important enteric pathogens using a magnetic bead based DNA microarray. World J. Microbiol. Biotechnol. 27, 163–169.

Sun, Y.-S., 2015. Use of Microarrays as a High-Throughput Platform for Label-Free Biosensing. J. Lab. Autom. 20, 334–53.

Swai, E.S., French, N.P., Karimuribo, E.D., Fitzpatrick, J.L., Bryant, M.J., Kambarage, D.M., Ogden, N.H., 2007. Prevalence and determinants of Cryptosporidium spp. infection in smallholder dairy cattle in Iringa and Tanga Regions of Tanzania. Onderstepoort J. Vet. Res. 74, 23–9.

Swiatek, D.L., Palombo, E. A, Lee, A, Coventry, M.J., Britz, M.L., Kirkwood, C.D., 2010. Detection and analysis of bovine rotavirus strains circulating in Australian calves during 2004 and 2005. Vet. Microbiol. 140, 56–62.

Szelei, J., Liu, K., Li, Y., Fernandes, S., Tijssen, P., 2010. Parvovirus 4-like virus in blood products. Emerg. Infect. Dis. 16, 561–564.

Talafha, A.Q., Hirche, S.M., Ababneh, M.M., Al-Majali, A.M., Ababneh, M.M., 2009. Prevalence and risk factors associated with bovine viral diarrhea virus infection in dairy herds in Jordan. Trop. Anim. Health Prod. 41, 499–506.

Tanabe, S., Hase, M., Yano, T., Sato, M., Fujimura, T., Akiyama, H., 2007. A real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods. Biosci. Biotechnol. Biochem. 71, 3131–5.

Tang, Y.J., Gumerlock, P.H., Weiss, J.B., Silva, J., 1994. Specific detection of Clostridium difficile toxin A gene sequences in clinical isolates. Mol. Cell. Probes 8, 463–467.

Taroncher-Oldenburg, G., Griner, E.M., Francis, C.A., Ward, B.B., 2003. Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl. Environ. Microbiol. 69, 1159–1171.

Tattersall, P., Cawte, P.J., Shatkin, A.J., Ward, D.C., 1976. Three structural polypeptides coded for by minite virus of mice, a parvovirus. J. Virol. 20, 273–89.

Tauxe, R., Pavia, A., 1998. Salmonellosis: nontyphoidal. In: Evans, A., Brachman, P. (Eds.), Bacterial Infections of Humans: Epidemiology and Control. Plenum Medical Book Co, pp. 613–630.

Tennant, S.M., Diallo, S., Levy, H., Livio, S., Sow, S.O., Tapia, M., Fields, P.I., Mikoleit,

M., Tamboura, B., Kotloff, K.L., Nataro, J.P., Galen, J.E., Levine, M.M., 2010. Identification by PCR of Non-typhoidal Salmonella enterica Serovars Associated with Invasive Infections among Febrile Patients in Mali. PLoS Negl. Trop. Dis. 4, e621.

Thao, N.T.T., Ngoc, N.T.K., Tú, P.V., Thúy, T.T., Cardosa, M.J., McMinn, P.C., Phuektes, P., 2010. Development of a multiplex polymerase chain reaction assay for simultaneous identification of human enterovirus 71 and coxsackievirus A16. J. Virol. Methods 170, 134–9.

Theuns, S., Vyt, P., Desmarets, L.M.B., Roukaerts, I.D.M., Heylen, E., Zeller, M., Matthijnssens, J., Nauwynck, H.J., 2016. Presence and characterization of pig group A and C rotaviruses in feces of Belgian diarrheic suckling piglets. Virus Res. 213, 172–183.

Thimmasandra Narayanappa, A., Sooryanarain, H., Deventhiran, J., Cao, D., Ammayappan Venkatachalam, B., Kambiranda, D., LeRoith, T., Heffron, C.L.C., Lindstrom, N., Hall, K., Jobst, P., Sexton, C., Meng, X.X.-J., Elankumaran, S. 2015. A novel pathogenic Mammalian orthoreovirus from diarrheic pigs and Swine blood meal in the United States. MBio 6, e00593–15.

Thoerner, P., Bin Kingombe, C.I., Bögli-Stuber, K., Bissig-Choisat, B., Wassenaar, T.M., Frey, J., Jemmi, T., 2003. PCR detection of virulence genes in Yersinia enterocolitica and Yersinia pseudotuberculosis and investigation of virulence gene distribution. Appl. Environ. Microbiol. 69, 1810–6.

Thomas, C.M., Nielsen, K.M., 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3, 711–21.

Thompson, R.C.A., 2004. The zoonotic significance and molecular epidemiology of Giardia and giardiasis. Vet. Parasitol. 126, 15–35.

Thomson, J.R., Smith, W.J., Murray, B.P., McOrist, S., 1997. Pathogenicity of three strains of Serpulina pilosicoli in pigs with a naturally acquired intestinal flora. Infect. Immun. 65, 3693–700.

Thomson, N.R., Clayton, D.J., Windhorst, D., Vernikos, G., Davidson, S., Churcher, C., Quail, M.A., Stevens, M., Jones, M.A., Watson, M., Barron, A., Layton, A., Pickard, D., Kingsley, R.A., Bignell, A., Clark, L., Harris, B., Ormond, D., Abdellah, Z., Brooks, K., Cherevach, I., Chillingworth, T., Woodward, J., Norberczak, H., Lord, A., Arrowsmith, C., Jagels, K., Moule, S., Mungall, K., Sanders, M., Whitehead, S., Chabalgoity, J.A., Maskell, D., Humphrey, T., Roberts, M., Barrow, P.A., Dougan, G., Parkhill, J., 2008. Comparative genome analysis of Salmonella Enteritidis PT4 and Salmonella Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. Genome Res. 18, 1624–1637.

Tijssen, P, Agbandje-McKenna, M, Almendral, JM, Bergoin, M, Flegel, TW, Hedman, K,

Kleinschmidt, JA, Li, D, Pintel, D, Tattersall, P., 2011. Parvoviridae. In: King, MQ, Adams, MJ, Carstens, E., Lefkowitz, E. (Ed.), Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, pp. 405–425.

Tischer, I., Mields, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. 91, 271–6.

Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of papovavirus-and picornavirus-like particles in permanent pig kidney cell lines. Zentralblatt für Bakteriol. Parasitenkunde, Infekt. und Hyg. Erste Abteilung Orig. R. A Medizinische Mikrobiol. und Parasitol. 226, 153–67.

Todd, D., Bendinelli, M., Biagini, P., Hino, S., Mankertz, A., M., S., Niel, C., Okamoto, H., Raidal, S., Ritchie, B.W., Teo, G.., 2005. Virus taxonomy, Eighth report of the International Committee for the Taxonomy of Viruses. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, B. LA (Ed.), Circoviridae. San Diego, pp. 327–334.

Todd, D., 2004. Avian circovirus diseases: lessons for the study of PMWS. Vet. Microbiol. 98, 169–174.

Todd, D., Niagro, F.D., Ritchie, B.W., Curran, W., Allan, G.M., Lukert, P.D., Latimer, K.S., Steffens, W.L., McNulty, M.S., 1991. Comparison of three animal viruses with circular single-stranded DNA genomes. Arch. Virol. 117, 129–35.

Toma, S., Deidrick, V.R., 1975. Isolation of Yersinia enterocolitica from swine. J. Clin. Microbiol. 2, 478–81.

Torres, A., 2004. Prevalence survey of Isospora suis in twelve European countries. In: Proceedings of the 18th Congress of the International Pig Veterinary Society, Hamburg, Germany, p. 243.

Torsein, M., Lindberg, A., Sandgren, C.H., Waller, K.P., Törnquist, M., Svensson, C., 2011. Risk factors for calf mortality in large Swedish dairy herds. Prev. Vet. Med. 99, 136–147.

Toze, S., 1999. PCR and the detection of microbial pathogens in water and wastewater. Water Res. 33, 3545–3556.

Trojnar, E., Otto, P., Roth, B., Reetz, J., Johne, R., 2010. The genome segments of a group D rotavirus possess group A-like conserved termini but encode group-specific proteins. J. Virol. 84, 10254–65.

Trott, D.J., Stanton, T.B., Jensen, N.S., Duhamel, G.E., Johnson, J.L., Hampson, D.J., 1996. Serpulina pilosicoli sp. nov., the agent of porcine intestinal spirochetosis. Int. J. Syst. Bacteriol. 46, 206–15.

Tseng, C.-H., Tsai, H.-J., 2007. Sequence analysis of a duck picornavirus isolate

indicates that it together with porcine enterovirus type 8 and simian picornavirus type 2 should be assigned to a new picornavirus genus. Virus Res. 129, 104–14.

Tsunemitsu, H., Jiang, B., Saif, L.J., 1992. Detection of group C rotavirus antigens and antibodies in animals and humans by enzyme-linked immunosorbent assays. J. Clin. Microbiol. 30, 2129–34.

Tsunemitsu, H., Kamiyama, M., Kawashima, K., Katsuda, K., Kohmoto, M., Saif, L.J., Shouji, T., Onodera, T., 2005. Molecular characterization of the major capsid protein VP6 of bovine group B rotavirus and its use in seroepidemiology. J. Gen. Virol. 86, 2569–75.

Tzipori, S., 1983. Cryptosporidiosis in animals and humans. Microbiol. Rev. 47, 84–96.

Tzipori, S., Chandler, D., Makin, T., Smith, M., 1980. Escherichia coli and rotavirus infections in four-week-old gnotobiotic piglets fed milk or dry food. Aust. Vet. J. 56, 279–84.

Tzipori, S., Gunzer, F., Donnenberg, M.S., De Montigny, L., Kaper, J.B., Donohue- Rolfe, A., Donohue-Rolfe, A., 1995. The role of the eaeA gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic Escherichia coli infection. Infect. Immun. 63, 3621–3627.

Uhde, F.L., Kaufmann, T., Sager, H., Albini, S., Zanoni, R., Schelling, E., Meylan, M., 2008. Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. Vet. Rec. 163, 362–6.

USDA, 2007. Dairy 2007 Part II: Changes in the U.S. Dairy Cattle Industry, 1991–2007. Fort Collins USDA-APHIS-VS, CEAH 57 – 61.

Uttamchandani, M., Neo, J.L., Ong, B.N.Z., Moochhala, S. 2009. Applications of microarrays in pathogen detection and biodefence. Trends Biotechnol. 27, 53–61.

Valat, C., Forest, K., Auvray, F., Métayer, V., Méheut, T., Polizzi, C., Gay, E., Haenni, M., Oswald, E., Madec, J.-Y., 2014. Assessment of Adhesins as an Indicator of Pathovar-Associated Virulence Factors in Bovine Escherichia coli. Appl. Environ. Microbiol. 80, 7230–4.

Van de Peer, Y., Chapelle, S., De Wachter, R., 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res. 24, 3381–3391.

Van Dung, N., Anh, P.H., Van Cuong, N., Hoa, N.T., Carrique-Mas, J., Hien, V.B., Campbell, J., Baker, S., Farrar, J., Woolhouse, M.E., Bryant, J.E., Simmonds, P., 2014. Prevalence, genetic diversity and recombination of species G enteroviruses infecting pigs in Vietnam. J. Gen. Virol. 95, 549–56.

van Rijn, P. a, van Gennip, H.G., Leendertse, C.H., Bruschke, C.J., Paton, D.J.,

Moormann, R.J., van Oirschot, J.T., 1997. Subdivision of the pestivirus genus based on envelope glycoprotein E2. Virology 237, 337–48.

Vandamme, P., On, S.L., 2001. Recommendations of the subcommittee on the taxonomy of Campylobacter and related bacteria. Int. J. Syst. Evol. Microbiol. 51, 719–21.

Vaughn, E.M., Halbur, P.G., Paul, P.S., 1995. Sequence comparison of porcine respiratory coronavirus isolates reveals heterogeneity in the S, 3, and 3-1 genes. J. Virol. 69, 3176–84.

Vemulapalli, R., Gulani, J., Santrich, C., 2009. A real-time TaqMan RT-PCR assay with an internal amplification control for rapid detection of transmissible gastroenteritis virus in swine fecal samples. J. Virol. Methods 162, 231–5.

Venu, R., Latha, B.R., Basith, S.A., Sreekumar, C., Raj, G.D., Raman, M., 2013. Factors influencing on prevalence of Cryptosporidium infection in south Indian dairy calves. J. Parasit. Dis. 37, 168–72.

Verma, H., Mor, S.K., Abdel-Glil, M.Y., Goyal, S.M., 2013. Identification and molecular characterization of porcine kobuvirus in U. S. swine. Virus Genes 46, 551–553.

Vilcek, S., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Paifi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch Virol 146, 99–115.

Virtala, A.M., Mechor, G.D., Gröhn, Y.T., Erb, H.N., 1996. Morbidity from nonrespiratory diseases and mortality in dairy heifers during the first three months of life. J. Am. Vet. Med. Assoc. 208, 2043–6.

Volokhov, D., Rasooly, A., Chumakov, K., Chizhikov, V., 2002. Identification of Listeria species by microarray-based assay. J. Clin. Microbiol. 40, 4720–4728.

Vora, G.J., Meador, C.E., Stenger, D. a., Andreadis, J.D., 2004. Nucleic Acid Amplification Strategies for DNA Microarray-Based Pathogen Detection. Appl. Environ. Microbiol. 70, 3047–3054.

Voth, D.E., Ballard, J.D., 2005. Clostridium difficile Toxins: Mechanism of Action and Role in Disease. Clin. Microbiol. Rev. 18, 247–263.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J.E., Dahbi, G., Mora, A., Lopez, C., Gonzalez, E.A., Blanco, J., 2007. Serotypes, virulence genes, intimin types and PFGE profiles of Escherichia coli isolated from piglets with diarrhoea in Slovakia. Vet. J. 174, 176–187.

Wageck Canal, C., Strasser, M., Hertig, C., Masuda, A., Peterhans, E., 1998. Detection of antibodies to bovine viral diarrhoea virus (BVDV) and characterization of

genomes of BVDV from Brazil. Vet. Microbiol. 63, 85–97.

Wagner, C., Polke, M., Gerlach, R.G., Linke, D., Stierhof, Y.D., Schwarz, H., Hensel, M., 2011. Functional dissection of SiiE, a giant non-fimbrial adhesin of Salmonella enterica. Cell. Microbiol. 13, 1286–1301.

Wales, A. D., Woodward, M.J., Pearson, G.R., 2005. Attaching-effacing bacteria in animals. J. Comp. Pathol. 132, 1–26.

Wang, B., Tian, Z.-J., Gong, D.-Q., Li, D.-Y., Wang, Y., Chen, J.-Z., An, T.-Q., Peng, J.-M., Tong, G.-Z., 2010. Isolation of serotype 2 porcine teschovirus in China: evidence of natural recombination. Vet. Microbiol. 146, 138–43.

Wang, D., Coscoy, L., Zylberberg, M., Avila, P.C., Boushey, H. a, Ganem, D., DeRisi, J.L., 2002. Microarray-based detection and genotyping of viral pathogens. Proc. Natl. Acad. Sci. U. S. A. 99, 15687–92.

Wang, G., Clark, C.G., Taylor, T.M., Pucknell, C., Barton, C., Price, L., Woodward, D.L., Rodgers, F.G., 2002. Colony multiplex PCR assay for identification and differentiation of Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, and C. fetus subsp. fetus. J. Clin. Microbiol. 40, 4744–7.

Wang, L., Ge, C., Wang, D., Li, Y., Hu, J., 2013. The survey of porcine teschoviruses, porcine circovirus and porcine transmissible gastroenteritis virus infecting piglets in clinical specimens in China. Trop. Anim. Health Prod. 45, 1087–91.

Wang, Q., Wang, S., Beutin, L., Cao, B., Feng, L., Wang, L., 2010. Development of a DNA microarray for detection and serotyping of enterotoxigenic Escherichia coli. J. Clin. Microbiol. 48, 2066–74.

Wang, Q.-H., Costantini, V., Saif, L.J., 2007. Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. Vaccine 25, 5453–66.

Wang, Q.H., Han, M.G., Funk, J.A., Bowman, G., Janies, D.A., Saif, L.J., 2005a. Genetic diversity and recombination of porcine sapoviruses. J. Clin. Microbiol. 43, 5963–5972.

Wang, Q.H., Myung, G.H., Cheetham, S., Souza, M., Funk, J.A., Saif, L.J., 2005b. Porcine noroviruses related to human noroviruses. Emerg. Infect. Dis. 11, 1874–1881.

Wang, Q.-H., Souza, M., Funk, J.A., Zhang, W., Saif, L.J., 2006. Prevalence of noroviruses and sapoviruses in swine of various ages determined by reverse transcription-PCR and microwell hybridization assays. J. Clin. Microbiol. 44, 2057–62.

Wang, R., Huang, J., Zhang, W., Lin, G., Lian, J., Jiang, L., Lin, H., Wang, S., Wang, S., 2011. Detection and identification of Vibrio parahaemolyticus by multiplex PCR

and DNA–DNA hybridization on a microarray. J. Genet. Genomics 38, 129–135.

Wang, X., Bai, A., Zhang, J., Kong, M., Cui, Y., Ma, X., Ai, X., Tang, Q., Cui, S., 2014. A new nanoPCR molecular assay for detection of porcine bocavirus. J. Virol. Methods 202, 106–111.

Wang, Z., Vora, G.J., Stenger, D.A., 2004. Detection and genotyping of Entamoeba histolytica, Entamoeba dispar, Giardia lamblia, and Cryptosporidium parvum by oligonucleotide microarray. J. Clin. Microbiol. 42, 3262–71.

Ward, L.A., Wang, Y., 2001. Rapid methods to isolate Cryptosporidium DNA from frozen feces for PCR. Diagn. Microbiol. Infect. Dis. 41, 37–42.

Wassenaar, T.M., 1997. Toxin production by Campylobacter spp. Clin. Microbiol. Rev. 10, 466–76.

Watanabe, Y., Millward, S., Graham, A.F., 1968. Regulation of transcription of the reovirus genome. J. Mol. Biol. 36, 107–123.

Wattiau, P., Weijers, T., Andreoli, P., Schliker, C., Veken, H. Vander, Maas, H.M.E., Verbruggen, A.J., Heck, M.E.O.C., Wannet, W.J., Imberechts, H., Vos, P., 2008. Evaluation of the Premi®Test Salmonella, a commercial low-density DNA microarray system intended for routine identification and typing of Salmonella enterica. Int. J. Food Microbiol. 123, 293–298.

Weinstock, G.M., 2012. Genomic approaches to studying the human microbiota. Nature 489, 250–6.

Weiss, M., Steck, F., Horzinek, M.C., 1983. Purification and partial characterization of a new enveloped RNA virus (Berne virus). J. Gen. Virol. 64 (Pt 9), 1849–58.

Weiss, S.R., Navas-Martin, S., 2005. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol. Mol. Biol. Rev. 69, 635–64.

Weldon, S.L., Blue, J.L., Wooley, R.E., Lukert, P.D., 1979. Isolation of picornavirus from feces and semen from an infertile bull. J. Am. Vet. Med. Assoc. 174, 168–9.

Weng, X.G., Song, Q.J., Wu, Q., Liu, M.C., Wang, M.L., Wang, J.F. 2015. Genetic characterization of bovine viral diarrhea virus strains in Beijing, China and innate immune responses of peripheral blood mononuclear cells in persistently infected dairy cattle. J. Vet. Sci. 16, 491.

Wenge, J., Steinhöfel, I., Heinrich, C., Coenen, M., Bachmann, L., 2014. Water and concentrate intake, weight gain and duration of diarrhea in young suckling calves on different diets. Livest. Sci. 159, 133–140.

Werling, D., Ruryk, A., Heaney, J., Moeller, E., Brownlie, J., 2005. Ability to differentiate between cp and ncp BVDV by microarrays: Towards an application in clinical

veterinary medicine? Vet. Immunol. Immunopathol. 108, 157–164.

Wick, L.M., Rouillard, J.M., Whittam, T.S., Gulari, E., Tiedje, J.M., Hashsham, S.A., 2006. On-chip non-equilibrium dissociation curves and dissociation rate constants as methods to assess specificity of oligonucleotide probes. Nucleic Acids Res. 34, e26.

Wieler, L.H., Ilieff, A., Herbst, W., Bauer, C., Vieler, E., Bauerfeind, R., Failing, K., Klös, H., Wengert, D., Baljer, G., Zahner, H., 2001. Prevalence of enteropathogens in suckling and weaned piglets with diarrhoea in southern Germany. J. Vet. Med. B. Infect. Dis. Vet. Public Health 48, 151–9.

Will, L.A., Paul, P.S., Proescholdt, T.A., Aktar, S.N., Flaming, K.P., Janke, B.H., Sacks, J., Lyoo, Y.S., Hill, H.T., Hoffman, L.J., 1994. Evaluation of rotavirus infection and diarrhea in Iowa commercial pigs based on an epidemiologic study of a population represented by diagnostic laboratory cases. J. Vet. Diagn. Invest. 6, 416–22.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Wilson, W.J., Strout, C.L., DeSantis, T.Z., Stilwell, J.L., Carrano, A. V, Andersen, G.L., 2002. Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. Mol. Cell. Probes 16, 119–27.

Wise, M.G., Siragusa, G.R., 2005. Quantitative detection of Clostridium perfringens in the broiler fowl gastrointestinal tract by real-time PCR. Appl. Environ. Microbiol. 71, 3911–6.

Witchell, T.D., Hoke, D.E., Bulach, D.M., Coutts, S.A.J., Driesen, S.J., Cordwell, S.J., Adler, B., 2011. The major surface Vsp proteins of Brachyspira hyodysenteriae form antigenic protein complexes. Vet. Microbiol. 149, 157–162.

Wong, C.W., Heng, C.L.W., Wan Yee, L., Soh, S.W.L., Kartasasmita, C.B., Simoes, E. a F., Hibberd, M.L., Sung, W.-K., Miller, L.D., 2007. Optimization and clinical validation of a pathogen detection microarray. Genome Biol. 8, R93.

Wood, C.N., 1969. Transmissible gastro-enteritis of swine. Vet. Bull. 39, 239–248.

Woode, G.N., Bridger, J.C., 1978. Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. J. Med. Microbiol. 11, 441–52.

Worliczek, H.L., Gerner, W., Joachim, A., Mundt, H.-C., Saalmüller, A., 2009. Porcine Coccidiosis – Investigations on the Cellular Immune Response against Isospora suis. Parasitol. Res. 105, 151–156.

Wray, C., Roeder, P.L., 1987. Effect of bovine virus diarrhoea-mucosal disease virus infection on salmonella infection in calves. Res. Vet. Sci. 42, 213–8.

Wu, Y.-C., Lee, J.-J., Tsai, B.-Y., Liu, Y.-F., Chen, C.-M., Tien, N., Tsai, P.-J., Chen, T.-H., 2016. Potentially hypervirulent Clostridium difficile PCR ribotype 078 lineage isolates in pigs and possible implications for humans in Taiwan. Int. J. Med. Microbiol. 306, 115–122.

Xia, H., Liu, L., Wahlberg, N., Baule, C., Belák, S., 2007. Molecular phylogenetic analysis of bovine viral diarrhoea virus: A Bayesian approach. Virus Res. 130, 53–62.

Xia, X.-Q., Jia, Z., Porwollik, S., Long, F., Hoemme, C., Ye, K., Müller-Tidow, C., McClelland, M., Wang, Y., 2010. Evaluating oligonucleotide properties for DNA microarray probe design. Nucleic Acids Res. 38, e121.

Xiao, C.-T., Gerber, P.F., Giménez-Lirola, L.G., Halbur, P.G., Opriessnig, T., 2013. Characterization of porcine parvovirus type 2 (PPV2) which is highly prevalent in the USA. Vet. Microbiol. 161, 325–330.

Xiao, C.T., Giménez-Lirola, L.G., Gerber, P.F., Jiang, Y.H., Halbur, P.G., Opriessnig, T., 2013. Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types. J. Gen. Virol. 94, 570–582.

Xiao, C.-T., Giménez-Lirola, L.G., Halbur, P.G., Opriessnig, T., 2012. Increasing porcine PARV4 prevalence with pig age in the U.S. pig population. Vet. Microbiol. 160, 290–296.

Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: an update. Exp. Parasitol. 124, 80–9.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R.C., Fayer, R., Lal, A.A., 1999. Genetic diversity within Cryptosporidium parvum and related Cryptosporidium species. Appl. Environ. Microbiol. 65, 3386–91.

Xie, Z., Fan, Q., Liu, J., Pang, Y., Deng, X., Xie, Z., Liji, X., Khan, M.I., 2012. Reverse transcription loop-mediated isothermal amplification assay for rapid detection of Bovine Rotavirus. BMC Vet. Res. 8, 133.

Xu, L.Z., Larzul, D., 1991. The polymerase chain reaction: Basic methodology and applications. Comp. Immunol. Microbiol. Infect. Dis. 14, 209–221.

Xue, F., Zhu, Y.M., Li, J., Zhu, L.C., Ren, X.G., Feng, J.K., Shi, H.F., Gao, Y.R., 2010. Genotyping of bovine viral diarrhea viruses from cattle in China between 2005 and 2008. Vet. Microbiol. 143, 379–383.

Yaeger, M., Funk, N., Hoffman, L., 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including Clostridium difficile and porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 14, 281–7.

Yamashita, T., Ito, M., Kabashima, Y., Tsuzuki, H., Fujiura, A., Sakae, K., 2003. Isolation

and characterization of a new species of kobuvirus associated with cattle. J. Gen. Virol. 84, 3069–3077.

Yamashita, T., Kobayashi, S., Sakac, K., Nakata, S., Chiba, S., Ishihara, Y., 1991. Isolation of Cytopathic Small Round Viruses with BS-C-l Cells from Patients with Gastroenteritis, The Journal of infectious diseases.

Yang, F., Liu, X., Zhou, Y., Lyu, W., Xu, S., Xu, Z., Zhu, L., 2015. Histopathology of Porcine kobuvirus in Chinese piglets. Virol. Sin. 30, 396–9.

Yang, G., Erdman, D.E., Kodani, M., Kools, J., Bowen, M.D., Fields, B.S., 2011. Comparison of commercial systems for extraction of nucleic acids from DNA/RNA respiratory pathogens. J. Virol. Methods 171, 195–199.

Yao, D., Kim, J., Yu, F., Nielsen, P.E., Sinner, E.-K., Knoll, W., 2005. Surface density dependence of PCR amplicon hybridization on PNA/DNA probe layers. Biophys. J. 88, 2745–51.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13, 134.

Yin, H., Li, Q., Liu, L., Chen, J., Wang, L., Liu, Z., 2012. Detection of Salmonella choleraesuis by Loop-mediated Isothermal Amplification. In: 2012 International Conference on Biomedical Engineering and Biotechnology. IEEE, pp. 999–1002.

You, M., 2014. Detection of four important Eimeria species by multiplex PCR in a single assay. Parasitol. Int.

Younis, E.E., Ahmed, A.M., El-Khodery, S.A., Osman, S.A., El-Naker, Y.F.I., 2009. Molecular screening and risk factors of enterotoxigenic Escherichia coli and Salmonella spp. in diarrheic neonatal calves in Egypt. Res. Vet. Sci. 87, 373–379.

Yu, G., Greninger, A.L., Isa, P., Phan, T.G., Martínez, M.A., de la Luz Sanchez, M., Contreras, J.F., Santos-Preciado, J.I., Parsonnet, J., Miller, S., DeRisi, J.L., Delwart, E., Arias, C.F., Chiu, C.Y., 2012. Discovery of a novel polyomavirus in acute diarrheal samples from children. PLoS One 7, e49449.

Yu, J., Jin, M., Zhang, Q., Li, H., Li, D., Xu, Z., Li, J., Cui, S., Yang, S., Liu, N., Duan, Z., 2009. Candidate porcine Kobuvirus, China. Emerg. Infect. Dis. 15, 823–5.

Yu, J.M., Xu, Z. qian, Li, B.W., Zhang, Q., Cui, S. xian, Jin, M., Duan, Z. jun, 2011. Analysis and characterization of the complete genome of a member of a new species of kobuvirus associated with swine. Arch. Virol. 156, 747–751.

Yutin, N., Galperin, M.Y., 2013. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environ. Microbiol. 15, 2631–2641.

Żak, M., Ad, P.Z., E, M.B., E, A.A.Z., E, R.M., E, B.K., 2011. Development of a miniaturized DNA microarray for identification of 66 virulence genes of Legionella pneumophila Opracowanie miniaturowej mikromacierzy DNA do identyfikacji 66 genów wirulencji Legionella pneumophila 838–848.

Zell, R., Dauber, M., Krumbholz, A., Henke, A., Birch-Hirschfeld, E., Stelzner, A., Prager, D., Wurm, R., 2001. Porcine teschoviruses comprise at least eleven distinct serotypes: molecular and evolutionary aspects. J. Virol. 75, 1620–31.

Zell, R., Krumbholz, A, Henke, a, Birch-Hirschfeld, E., Stelzner, a, Doherty, M., Hoey, E., Dauber, M., Prager, D., Wurm, R., 2000. Detection of porcine enteroviruses by nRT-PCR: differentiation of CPE groups I-III with specific primer sets. J. Virol. Methods 88, 205–18.

Zell, R., Krumbholz, A., Dauber, M., Hoey, E., Wutzler, P., 2006. Molecular-based reclassification of the bovine enteroviruses. J. Gen. Virol. 87, 375–85.

Zeng, S., Wang, D., Fang, L., Ma, J., Song, T., Zhang, R., Chen, H., Xiao, S., 2011. Complete coding sequences and phylogenetic analysis of porcine bocavirus. J. Gen. Virol. 92, 784–788.

Zhai, L., Kong, X., Lu, Z., Lv, F., Zhang, C., Bie, X., 2014. Detection of Salmonella enterica serovar Dublin by polymerase chain reaction in multiplex format. J. Microbiol. Methods 100, 52–57.

Zhai, S., Yue, C., Wei, Z., Long, J., Ran, D., Lin, T., Deng, Y., Huang, L., Sun, L., Zheng, H., Gao, F., Zheng, H., Chen, S., Yuan, S., 2010. High prevalence of a novel porcine bocavirus in weanling piglets with respiratory tract symptoms in China. Arch. Virol. 155, 1313–7.

Zhang, C., Liu, L., Wang, P., Liu, S., Lin, W., Hu, F., Wu, W., Chen, W., Cui, S., 2011. A potentially novel reovirus isolated from swine in northeastern China in 2007. Virus Genes 43, 342–9.

Zhang, C.F., Cui, S.J., Hu, S., Zhang, Z., Guo, Q., Zell, R., 2010. Isolation and characterization of the first Chinese strain of porcine Teschovirus-8. J. Virol. Methods 167, 208–213.

Zhang, H.B., Huang, L., Liu, Y.J., Lin, T., Sun, C.Q., Deng, Y., Wei, Z.Z., Cheung, A.K., Long, J.X., Yuan, S.S., 2011. Porcine bocaviruses: genetic analysis and prevalence in Chinese swine population. Epidemiol. Infect. 139, 1581–6.

Zhang, Q., Zhang, C., Gao, M., He, X., Diao, Y., Goyal, S.M., Mor, S.K., Huang, J., 2015. Evolutionary, epidemiological, demographical, and geographical dissection of porcine bocavirus in China and America. Virus Res. 195, 13–24.

Zhang, W.-J., Xu, L.-H., Liu, Y.-Y., Xiong, B.-Q., Zhang, Q.-L., Li, F.-C., Song, Q.-Q., Khan, M.K., Zhou, Y.-Q., Hu, M., Zhao, J., 2012. Prevalence of coccidian infection in

suckling piglets in China. Vet. Parasitol. 190, 51–55.

Zhang, Y., Szustakowski, J., Schinke, M., 2009. Bioinformatics analysis of microarray data. Methods Mol. Biol. 573, 259–84.

Zhao, J., Tang, S., Storhoff, J., Marla, S., Bao, Y.P., Wang, X., Wong, E.Y., Ragupathy, V., Ye, Z., Hewlett, I.K. 2010. Multiplexed, rapid detection of H5N1 using a PCR-free nanoparticle-based genomic microarray assay. BMC Biotechnol. 10, 74.

Zhao, K., Shi, W., Han, F., Xu, Y., Zhu, L., Zou, Y., Wu, X., Zhu, H., Tan, F., Tao, S., Tang, X., 2011. Specific, simple and rapid detection of porcine circovirus type 2 using the loop-mediated isothermal amplification method. Virol. J. 8, 126.

Zhao, Z.-P., Yang, Z., Lin, W.-D., Wang, W.-Y., Yang, J., Jin, W.-J., Qin, A.-J., 2016. The rate of co-infection for piglet diarrhea viruses in China and the genetic characterization of porcine epidemic diarrhea virus and porcine kobuvirus. Acta Virol. 60, 55–61.

Zheng, D.-P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I., Monroe, S.S., 2006. Norovirus classification and proposed strain nomenclature. Virology 346, 312–323.

Zhou, W., Ullman, K., Chowdry, V., Reining, M., Benyeda, Z., Baule, C., Juremalm, M., Wallgren, P., Schwarz, L., Zhou, E., Pedrero, S.P., Hennig-Pauka, I., Segales, J., Liu, L., 2016. Molecular investigations on the prevalence and viral load of enteric viruses in pigs from five European countries. Vet. Microbiol. 182, 75–81.

Zhou, Y., Chen, L., Zhu, L., Xu, Z., 2013. Molecular detection of porcine torovirus in piglets with diarrhea in southwest China. ScientificWorldJournal. 2013, 984282.

Zhu, X., Wang, Y., Chen, J., Zhang, X., Shi, H., Shi, D., Gao, J., Feng, L., 2016. Development of TaqMan real-time reverse transcription-polymerase chain reaction for the detection and quantitation of porcine kobuvirus. J. Virol. Methods 234, 132–136.

Ziebuhr, J., 2004. Molecular biology of severe acute respiratory syndrome coronavirus. Curr. Opin. Microbiol. 7, 412–419.

Zlateva, K.T., Crusio, K.M., Leontovich, A.M., Lauber, C., Claas, E., Kravchenko, A. a., Spaan, W.J.M., Gorbalenya, A.E., 2011. Design and validation of consensus-degenerate hybrid oligonucleotide primers for broad and sensitive detection of corona- and toroviruses. J. Virol. Methods 177, 174–183.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–15.

Appendix I. Faecal samples

I.1. Porcine faecal samples with amount of DNA and RNA in ng/µl

Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA
Batch 1	PM1B278	1	-	-	Batch 3	PM9A495	9	48.45	38.64
	PM2A3	2	-	-		PM9A497	9'	17.94	14.41
	PM2A1	3	-	-		PM13A468	13	35.02	27.44
	PM2B269	4	93.91	75.35		PM15B69	15	10.63	9.14
	PM2A2	5	-	-		PM16A128	16	93.83	77.38
	PM2B266	6	-	-	Batch 4	PM4B77	A	28.86	22.01
	PM2B262	7	101.7	76.25		PM5A421	B	40.39	31.87
	PM3B1	8	-	-		PM16A137	C	40.44	33.47
	PM4A221	9	85.08	-		PM17B167	D	30.39	23.61
Batch 2	PM2B271	1	99.87	79.03		PM21A500	E	33.83	26.94
	PM4A231	2	113.4	89.49		PM22A216	F	24.79	21.58
	PM4B472	3	141.3	111		PM23A398	G	35.07	27.82
	PM5A416	4	111.3	89.29		PM23A407	H	36.19	28.12
	PM8B3	5	97.2	77.05		PM24A237	I	29.26	22.27
	PM8B48	6	105.1	84.34	Batch 5	PM5A417	5A	65.92	85.94
	PM17A416	7	96.26	79.48		PM9A1	9A	29.89	3.10
	PM17A418	8	128.6	102.2		PM15A444	15A	191.5	11.74
	PM17A420	9	109.3	86.35		PM15B1	15B	63.84	112.0
	PM17A421	10	110.5	89.47		PM17A422	17A	101.9	38.86
	PM17A428	11	114.4	90.09	Batch 6	PM8A2	1B	-	-
	PM18A447	12	98.79	78.06		PM18A450	2B	-	-
	PM18A451	13	108.5	84.7		PM8A3	3B	-	-
	PM21B157	14	104.8	83.51		PMB2271	4B	-	-
	PM23A392	15	96.97	77.47		PM1A1	6B	-	-
Batch 3	PM8A469	8A	38.17	30.28			7B	-	-
	PM8B44	8B	80.63	64.91					

-: not performed

I.2. Bovine faecal samples with amount of DNA and RNA in ng/µl

Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA
Batch 1	2bis	2b	145.8	42.28	Batch 2	1906	6	11.15	74.11	Batch 3	6/5	6/5	-	-	Batch 4	18T	18T	96.11	462.8
	6?	6?	94.93	1551		1907	7	125.1	195.4		91011	9	-	-		20T	20T	111.8	759.5
	11	11	11.45	1030		1908	8	51.91	249.2		12/07/01	12	-	-		25T	25T	117.9	367.1
	12	12	39.83	143.3		1909	9	34.67	237.7		12/07/02/4	12'	-	-		29T	29T	101.5	587.4
	13	13	230.7	254.1		1910	10	88.94	184.2		161111	161	-	-		30T	30T	88.48	648.4
	14	14	56.17	96.76		1911	11	28.37	157.5		162312	162	-	-		31T	31T	86.39	145.9
	26	26	36.67	200.2		1912	12	128.2	145.9		165022	165	-	-		35T	35T	70.90	222.5
	27	27	56.04	91.15		1913	13	20.15	140.5		190186	19.1	-	-		38T	38T	70.95	559.8
	37	37	77.96	163.3		1914	14	109.2	211.2		1905623	19.2	-	-		40T	40T	80.90	340.8
	58T	58	89.58	291.3	Batch 3	Bes 1	B1	-	-		1905624	19.3	-	-		41T	41T	81.24	485.7
	59	59	52.40	69.48		Bes 2	B2	-	-	Batch 4	3T	3T	100.2	353.2		42T	42T	83.58	294.3
	60T	60	24.08	196.3		HEM	Hem	-	-		4T	4T	165.9	174.6		43T	43T	136.5	201.3
	63T	63	14.67	116.9		1/3	1/3	-	-		6T	6T	150.8	571.2		45T	45T	142.3	371.3
Batch 2	1901	1	148.1	102		2/8	2/8	-	-		7T	7T	256.2	294.5	Batch 5	S1	S1	27.92	197.3
	1903	3	98.63	123.5		3/10	3/10	-	-		9T	9T	120.3	361.3		S2	S2	295.6	195.8
	1905	5	84.03	122.2		5/8	5/8	-	-		13T	13T	126.4	441.9		S3	S3	191.0	152.0

-: not performed

Continued

Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA	Batch No	Sample ID	Lab sample ID	DNA	RNA
Batch 5	S4	S4	31.27	89.98	**Batch 7**	C1	C1	968.9	117.2	**Batch 7**	C18	C18	108.6	106.6	**Batch 9**	44T	44T	41.58	73.47
	S5	S5	165.5	782.7		C2	C2	281.6	202.4	**Batch 8**	Va	Va	56.79	157.7		47	47	79.25	161.9
	S6	S6	146.6	252.8		C3	C3	232.6	212.8		Sva	Sva	42.75	157		49	49	265.1	565.7
Batch 6	1	1	340.7	188.3		C4	C4	162.6	232.3		V1	V1	185.7	135		53	53	247.5	473.3
	2	2	285.8	355.6		C5	C5	59.69	265.4		V2	V2	189.8	131.3		54	54	56.09	92.44
	3	3	270.7	268.6		C6	C6	59.99	168.5		Ve1	Ve1	96.87	86.28		55	55	32.99	48.74
	4	4	131.7	296.3		C7	C7	57.37	220.2		Ve2	Ve2	150.4	98.76	**Batch 10**	1Tl	1Tl	-	-
	5	5	138.6	122.6	**Batch 7**	C8	C8	463.6	295.2		3266	32	144.4	78.54		2Tl	2Tl	-	-
	6	6	135.6	189.1		C10	C10	22.04	641.7		9475	94	125.5	63.79		3Tl	3Tl	-	-
	A	A	230.7	912.1		C11	C11	117.7	422.9		1520960	15	141.3	117.4		4Tl	4Tl	-	-
	B	B	1262	938.5		C12	C12	605.5	266.0	**Batch 9**	Cam1	Cam1	132.2	123.1		5Tl	5Tl	-	-
	C	C	264.9	592.6		C13	C13	386.6	246.0		Cam2	Cam2	57.01	34.97		6Tl	6Tl	-	-
	D	D	220.4	496.5		C14	C14	565.5	693.5		Cam3	Cam3	98.41	73.37		7Tl	7Tl	-	-
	E	E	47.37	215.5		C15	C14	1259	655.7		Cam4	Cam4	95.82	220		8Tl	8Tl	-	-
	F	F	77.21	690.0		C16	C16	593.2	429.7		Cam5	Cam5	110.5	83.77					
	G	G	259.5	85.85		C17	C17	27.49	80.46		Cap	Cap	122.5	104.1					

-: not performed

Appendix II. Published primers sequences

Microrganism / Host	**Target gene**	**Forward primer sequence 5'->3'**	**Reverse primer sequence 5'->3'**
Bovine / Porcine kobuvirus	RdRp	ATGTTGTTRATGATGGTGTTGA	TGGAYTACAAGTGTTTTGATGC
Nebovirus	RdRp and capsid	GTGATTTAATTAGAGAAGGAAAC	CGTAGCAGCACTAGCCATA
Bovine/Porcine parvovirus	BParVgp1	CTACAATGATTTGGTGGTACATTT	TAGAAAGCATCATGACTAACCAGT
BVDV Generic	5'UTR, Npro, C	CTCTGCTGTACATGGCACATG	CGTCGAACCAGTGACGACT
Bovine /Porcine Rotavirus A	VP7	GGCTTTAAAAGAGAGAATTTCCGTCTGG	CTGGTCACATCATACAATTCTAATAAG
PEDV	Spike	TTCTGAGTCACGAACAGCCA	CATATGCAGCCTGCTCTGAA
TGEV	Spike	GTGGTTTTGGTYRTAAATGC	CACTAACCAACGTGGARCTA
Porcine circovirus 1 and 2	ORF1	GAGGTGGGTGTTCACCCT	CACACAGTCTCAGTAGATCATCC
Porcine Bocavirus	NS	ACAGGCAGCCGATCACTCACTAT	CTCGTTCCTCCCATCAGACACTT
Porcine parvovirus	NS1	AGTTAGAATAGGATGCGAGGAA	AGAGTCTGTTGGTGTATTTATTGG
Salmonella spp.	16S rRNA	TGTTGTGGTTAATAACCGCA	CACAAATCCATCTCTGGA
Escherichia coli	mdh	GGTATGGATCGTTCCGACCT	GGCAGAATGGTAACACCAGAGT
Clostridium spp.	16S rRNA	ACACGGTCCAAACTCCTACG	AGGCGAGTTTCAGCCTACAA
Clostridium spp.	*tcdA*	GCATGATAAGGCAACTTCAGTGG	GAGTAAGTTCCTCCTGCTCCATCAA
Clostridium spp.	*tcdB*	GGTG GAGCTTCAATTGGAGAG	GT GTAACCTACTITCATAACACCAG
Campylobacter spp.	16SrRNA	ATCTAATGGCTTAACCATTAAAC	GGACGGTAACTAGTTTAGTATT
Cryptosporidium spp.	18S rRNA	AAGCTCGTAGTTGGATTTCTG	TAAGGTGCTGAAGGAGTAAGG

Appendix III. Cycling conditions for published primers

Pathogen	Target gene	Product size (bp)	Gen-ome	Cycling conditions		No of cycles	Primer name	Author
Bovine/ Porcine kobuvirus	RdRp	216	ssRNA	In. Denat.	94°C for 3 min	40	Fwd : UNIV-kobu-F Rse : UNIV-kobu-R	Reuter et al. (2009)
				Denat.	94°C for 30 sec			
				Ann.	49°C for 1 min 30 sec			
				Ext.	72°C for 1 min			
				Fin.Ext.	72°C for 10 min			
Nebovirus	RdRp and Capsid	1669	ssRNA	In. Denat.	94°C for 2 min	35	Fwd: NBcap-F3 Rse : NBcap-R	Han et al. (2004)
				Denat.	94°C for 30 sec			
				Ann.	56°C for 30 sec			
				Ext.	72°C for 1 min 30 sec			
				Fin.Ext.	72°C for 10 min			
Bovine parvovirus	BparVgp 1	281	ssDNA	In. Denat.	95°C for 2 min	30	-	Bae et Kim (2010)
				Denat.	95°C for 1 min			
				Ann.	58°C for 30 sec			
				Ext.	72°C for 1 min			
				Fin.Ext.	72°C for 5 min			
BVDV	5'UTR, Npro and Capsid	1013	ssRNA	In. Denat.	-	45	Fwd: PanBVDVpcrF Rse: PanBVDVpcrRb	Decaro et al. (2012)
				Denat.	94°C for 30 sec			
				Ann.	50°C for 30 sec			
				Ext.	68°C for 1 min			
				Fin.Ext.	68°C for 10 min			

Fwd: Forward, Rse: Reverse

Continued

Pathogen	Target gene	Product size (bp)	Genome	Cycling conditions		No of cycles	Primer name	Author
Bovine / Porcine rotavirus A	VP7	1062	dsRNA	In. Denat.	-	35	Fwd: Beg9 Rse : End9	Gouvea et al. (1991)
				Denat.	94°C for 1 min			
				Ann.	45°C for 1 min			
				Ext.	72°C for 1 min			
				Fin.Ext.	72°C for 10 min			
PEDV	Spike	651	ssRNA	In. Denat.	94°C for 5 min	5	Fwd: P1 Rse : P2	Kim et al. (2001)
				Denat.	94°C for 30 sec			
				Ann.	55°C for 30 sec			
				Ext.	72°C for 30 sec			
				Denat.	94°C for 30 sec	30		
				Ann.	53°C for 30 sec			
				Ext.	72°C for 30 sec			
				Fin.Ext.	72°C for 7 min			
TGEV	Spike	859	ssRNA	In. Denat.	94°C for 5 min	5	Fwd: T1 Rse : T2	Kim et al. (2001)
				Denat.	94°C for 30 sec			
				Ann.	55°C for 30 sec			
				Ext.	72°C for 30 sec			
				Denat.	94°C for 30 sec	30		
				Ann.	53°C for 30 sec			
				Ext.	72°C for 30 sec			
				Fin.Ext.	72°C for 7 min			

Fwd: Forward, Rse: Reverse

Continued

Pathogen	Target gene	Product size (bp)	Genome	Cycling conditions		No of cycles	Primer name	Author
Porcine circovirus PCV1/2	ORF1	646	ssDNA	In. Denat.	-	35	Fwd: ORF1.PCV1.S2 Rse : ORF1.PCV1.AS6	Ouardani et al. (1999)
				Denat.	94°C for 1 min			
				Ann.	53°C for 1 min			
				Ext.	65°C for 3 min			
				Fin.Ext.	72°C for 7 min			
Porcine bocavirus	NS1	680	ssDNA	In. Denat.	94°C for 6 min	35	Fwd: PboV-F Rse : PboV-R	Liu et al. (2014)
				Denat.	94°C for 30 sec			
				Ann.	52°C for 30 sec			
				Ext.	68°C for 90 sec			
				Fin.Ext.	-			
Porcine parvovirus	NS1	265	ssDNA	In. Denat.	95°C for 5 min	30	Fwd: PPVF Rse : PPVR	Xu et al. (2012)
				Denat.	94°C for 30 sec			
				Ann.	56°C for 30 sec			
				Ext.	72°C for 45 sec			
				Fin.Ext.	72°C for 10 min			
Salmonella spp.	16S rRNA	574	DNA	In. Denat.	-	35	Fwd: 16SF1 Rse : 16SIII	Lin and Tsen (1996)
				Denat.	92°C for 20 sec			
				Ann.	56°C for 20 sec			
				Ext.	72°C for 30 sec			
				Fin.Ext.	72°C for 2 min			

Fwd: Forward, Rse: Reverse

Continued

Pathogen	Target gene	Product size (bp)	Genome	Cycling conditions		No of cycles	Primer name	Author
Escherichia coli	mdh	304	DNA	In. Denat.	94°C for 10 min	35	Fwd: mdh.269F Rse : mdh.530R	Tarr et al. (2002)
				Denat.	92°C for 30 sec			
				Ann.	55°C for 30 sec			
				Ext.	72°C for 45 sec			
				Fin.Ext.	72°C for 7 min			
Clostridium difficile	16S rRNA	900		In. Denat.	98°C for 30 sec	36	-	Darkoh et al. (2011)
				Denat.	98°C for 10 sec			
				Ann.	60°C for 10 sec			
				Ext.	72°C for 10 sec			
				Fin.Ext.	72°C for 1 min			
Clostridium difficile	Toxin A (*tcdA*)	634		In. Denat.	-	40	Fwd: YT-28 Rse: YT-29	Tang et al. (1994)
				Denat.	55°C for 10 min			
				Ann.	55°C for 30 sec			
				Ext.	72°C for 30 sec			
				Fin.Ext.	-			
Clostridium difficile	Toxin B (*tcdB*)	399		In. Denat.	-	40	Fwd: YT-17 Rse: YT-18	Gumerlock et al. (1993)
				Denat.	55°C for 10 min			
				Ann.	55°C for 30 sec			
				Ext.	72°C for 30 sec			
				Fin.Ext.	-			

Fwd: Forward, Rse: Reverse

Continued

Pathogen	Target gene	Product size (bp)	Gen-ome	Cycling conditions		No of cycles	Primer name	Author
Campylobacter spp.	16S rRNA	857	DNA	In. Denat.	95°C 10 min	35	Fwd: MD16S1 Rse : MD16S2	Denis et al. (1999)
				Denat.	95°C for 30 sec			
				Ann.	59°C for 1.5 min			
				Ext.	72°C for 1 min			
				Fin.Ext.	72°C for 10 min			
Cryptosporidium spp.	18S rRNA	435	DNA	In. Denat.	95°C 3 min	35	Fwd: CPB-DIAGF Rse : CPB-DIAGR	Johnson et al. (1995)
				Denat.	94°C for 45 sec			
				Ann.	55°C for 45 sec			
				Ext.	72°C for 1 min			
				Fin.Ext.	72°C for 7 min			

ssRNA: Single stranded RNA, ssDNA: Single stranded DNA, dsRNA: Double stranded RNA, In. Denat. : Initial denaturation, Denat.: Denaturation, Ann.: Annealing, Ext.: Extension, Fin.Ext.: Final extension, Fwd: Forward, Rse: Reverse.

Appendix IV. Designed primers

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp0	Gene
B_frag_bft_F	Forward	CCCTCTGGTAGTCTAGGCGT	1470	Metalloprotease enterotoxin
B_frag_bft_R	Reverse	CGCTGCTCTTTTTCCGTTCC		
B_frag_16S_F	Forward	AGGGGCATCAGGAAGAAAGC	1954	16S rRNA
B_frag_16S_R	Reverse	ACTTTCTTTTATCGCCAGGGA		
B_frag_cepA_F	Forward	CCCGGGTATACGGGACGA	1313	Beta-lactamase (cepA)
B_frag_cepA_R	Reverse	GTAGCCGGTTTTGTTATCGGG		
B_hyod_16S_F	Forward	TGGAGAGTTTGATTCTGGCTC	1494	16S rRNA
B_hyod_16S_R	Reverse	CACACCTTCCGGTACGGC		
B_hyod_nox_F	Forward	AAGCAACCATAGTCATCCAAGA	1320	NADH Oxidase
B_hyod_nox_R	Reverse	ACCATGCTGGTACATGGGC		
B_pilo_16S_F	Forward	AAAGGAGGTGATCCAGCCAC	1624	16S rRNA
B_pilo_16S_R	Reverse	AAGTTCGGAGACCTAAACGC		
B_Pilo_cut_F	Forward	TGATTTGCTAGAAGTGACAGAAAAA	1204	cutF
B_Pilo_cut_R	Reverse	ACTTTGAGGAACCCCAGGTG		
B_Pil_o137_F	Forward	GCTGTAGTTCCTGCTACAAAATGT	964	Class D beta-lactamase (OXA-137)
B_Pil_o137_R	Reverse	ACTCCGCAAAAATATCAAGGATTAT		
B_Pil_OX_F	Forward	ACTGAAGCTGTCCTTGCTGT	1165	Beta-lactamase
B_Pil_OX_R	Reverse	GGGCGGGCGTTAACATTTC		
B_sp_NADH_F	Forward	CTTTGGGTTGGCGGAGTAGT	919	NADH oxidase (nox)
B_sp_NADH_R	Reverse	CAGACCATCCAGTAGAAGCCA		
C_coli_16S_F	Forward	ATCCTGGCTCAGAGTGAACG	1450	16S rRNA
C_coli_16S_R	Reverse	CTTCACCCCAGTCGCTGATT		
C_col_ceuE_F	Forward	TGCAGCAGCGTTAGTTTTAGC	840	Enterochelin ABC transporter, periplasmic enterochelin-binding protein
C_col_ceuE_R	Reverse	AGCTTAATTTTAACAGCTTGCGA		
C_coli_alp3_F	Forward	TGCAAGGAACAGTGAATTGGAG	969	Aminoglycosidase aphA
C_coli_alp3_R	Reverse	TGGACAGTTGCGGATGTACT		
C_jj_1623S_F	Forward	GAATCAGCGACTGGGGTGAA	965	16S-23S intergenic spacer
C_jj_1623S_R	Reverse	AAGCTTCTTGACAGCTCCCC		
C_jej_cdt_1_F	Forward	AGCAGCTGTTAAAGGTGGGG	1142	Cytolethal distending toxin cdt
C_jej_cdt_1_R	Reverse	TGTGCAACAAGGTGGAACAC		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
C_jej_iam_F	Forward	CGTTGCCTGATGGAGTCGTT	502	Invasion associated marker (iamA and 420nte)
C_jej_iam_R	Reverse	TGTGGTAGACGAGCGATGTT		
C_jej_aadE_F	Forward	TCTGGAATGTAACCCCCTATGT	1498	Class I 420ntegrin aminoglycoside adenyltransferase (aadA2) and plasmid aadE
C_jej_aadE_R	Reverse	GTTCAGCTAAGCGGCTGTCT		
C_dif_16S_F	Forward	TAGATAATCGGCTTCGGGCG	1469	16S rRNA
C_dif_16S_R	Reverse	ACAAGAAACAAACCATAAAGCCAGA		
C_df_cdtAB_F	Forward	GGGAAGGACAAGCACTGTCT	1774	Binary toxin
C_df_cdtAB_R	Reverse	TTCCACTTACTTGTGTTGTTTCTAA		
C_dif_tcdA_2_F	Forward	TGCTATTGCTGCGACTCATCT	1437	Toxin A
C_dif_tcdA_2_R	Reverse	CCAGGGGCTTTTACTCCATCA		
C_dif_tcdB_3_F	Forward	TGAAGAGAACAAGGTATCACAAGT	1266	Toxin B
C_dif_tcdB_3_R	Reverse	ACAGTATTAGCTGGTGCAAAGT		
C_dif_gyAB_F	Forward	TGATGAACTGGGGTCTTTCCT	450	gyrase subunits (gyrA and gyrB)
C_dif_gyAB_R	Reverse	TGGGTCCATTCTACATCAGCA		
C_perf_16S_F	Forward	GACTTCACCCCAATCGCTGA	1427	16S rRNA
C_perf_16S_R	Reverse	GGCGTGCTTAACACATGCAA		
C_per_cpb2_F	Forward	TGAAAACAGAGTTTTTAAATGGTGC	415	plasmid-encoded beta 2 toxin cpb2
C_per_cpb2_R	Reverse	TTGAAAGTTTCTCCTGAACCTAGA		
C_perf_cpe_F	Forward	TCCAATGGTGTTCGAAAATGC	876	enterotoxin (cpe)
C_perf_cpe_R	Reverse	ACAAGAACATATTGTCCAGCATC		
C_per_ermB_F	Forward	TTAAGAAGATAGCGCACGAGT	960	methylase (ermBP) and methylase (ermQ)
C_per_ermB_R	Reverse	ATAGCCACAACCCGTGACTT		
EAEC_aggA_F	Forward	CGGGTTGAGAAGCGGTGTAA	729	Aggregative adherence fimbria aggA
EAEC_aggA_R	Reverse	GATCGAGGGGGATAAGCTGC		
EAEC_aggR_F	Forward	GCGAAAGCGGATGCGATATTT	718	Aggregative adherence fimbria aggR
EAEC_aggR_R	Reverse	ACCGCCCTAACGGAAAATTG		
EAEC_astA_F	Forward	TGCTGACTGAACTGAAAAACCG	220	Enteroaggregative heat-stable enterotoxin 1 (astA)
EAEC_astA_R	Reverse	TCTTCCGTGGGAGCCTGATA		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
EPEC_eae_F	Forward	AACATTATCGGGCAGCCGTT	1569	Intimin eae
EPEC_eae_R	Reverse	TTTTATTTTCCGGGATTTGAGATGT		
EPEC_bfp_F	Forward	CTTGGCACTTGCGTGTCTTT	1353	Bundle forming pili bfp
EPEC_bfp_R	Reverse	AAGTATGCAGAGCACACCCC		
ETEC_F4_F	Forward	TGGTTCGGTCGATATCGGTG	760	ETEC F4ac FaeG gene
ETEC_F4_R	Reverse	AGTTATTGCTACGTTCAGCGG		
ETEC_F5_F	Forward	CGTTGCAACCAGCTACACTG	1120	K99 fimbrial subunit
ETEC_F5_R	Reverse	ACTGACTTAGTCGCTCCCTG		
ETEC_F6_F	Forward	CGGAGCCCTAGAGCCAATTT	1760	fimbriae 987P subunit
ETEC_F6_R	Reverse	CAATGGCTAGTCGCCAAGGA		
ETEC_F18_F	Forward	TGTCTGTGTTTGCGTCTACTCT	848	F18 fimbrial adhesin
ETEC_F18_R	Reverse	CTCGAAAACAATGGGCACCG		
ETEC_Lta_F	Forward	CAACCTCTGACTGATAGTCTGAAAA	798	Heat labile toxin a
ETEC_Lta_R	Reverse	TGGCGTTATCTTTTTCCGGATTG		
ETEC_LTb_F	Forward	GCTCCCTGTAGTGGAAGCTG	642	Heat labile toxin b
ETEC_LTb_R	Reverse	GGAGACCCAGAATCTGAGCA		
ETEC_ST_F	Forward	ATAACGGAAGCCGCGTGTAT	886	Heat stable toxin
ETEC_ST_R	Reverse	CCGTGAAACAACATGACGGG		
ETEC_AntTx_F	Forward	ACAAGTCTGTCCGTTAAATGCC	686	toxin of toxin antitoxin
ETEC_AntTx_R	Reverse	TCTCAAAGGTGCGTTATGCCT		
STEC_ShgTx_F	Forward	CAGACCGGCAACAACTGACT	1375	Shiga toxin subunit A and shiga toxin subunit B
STEC_ShgTx_R	Reverse	CTTCAGGGGGTGGAGGATGT		
EC_ATB_aad_F	Forward	TGTACTGGTACTGGTTTCGGG	961	aadA and aadB
EC_ATB_aad_R	Reverse	AGTCTTCCCCAGCTCTCTAA		
EC_ATB_bla_F	Forward	AGACGTCAGGTGGCACTTTT	1139	Betalactamase
EC_ATB_bla_R	Reverse	TGACGCTCAGTGGAACGAAA		
EC_AT_dfrA_F	Forward	ACGCCTAGAGATGCTTGTTT	957	Class I integron
EC_AT_dfrA_R	Reverse	AGGGGCTCACACTTCTGGTA		
EC_AT_ereA_F	Forward	TCTGTGAGCCGGGTTATTGG	1326	Class II 421ntegrin multidrug resistance
EC_AT_ereA_R	Reverse	TTCGCAGGTTATGCTCCCTC		
EC_ATB_vim_F	Forward	GTTCAAACACGCCAGGCATT	961	Resistance genes
EC_ATB_vim_R	Reverse	CATTCAGCCAGTTATGCCGC		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
L_intr_16S_F	Forward	GGCGTGCTTAACACATGCAA	1491	16S rRNA
L_intr_16S_R	Reverse	GTTCCCCTACGGCTACCTTG		
L_intr_16S_F	Forward	GGCGTGCTTAACACATGCAA	1491	16S rRNA
L_intr_16S_R	Reverse	GTTCCCCTACGGCTACCTTG		
L_int_fliM_3_F	Forward	TCCCTACAGCTTGAAGTCTTG	1499	Flagellar biosynthesis protein flhA
L_int_fliM_3_R	Reverse	TGCAATGGATGGTGCAGGTA		
L_int_T3SS_1_F	Forward	GCCATTTCTTCAGCTGTCTGG	1069	Type 3 secretion system
L_int_T3SS_1_R	Reverse	ATGGTGCAGCCAGTGTCTTT		
S_chl_GMx_1_F	Forward	CTTGCTGCTTTGCTGACGAG	1460	16S rRNA
S_chl_GMx_1_R	Reverse	TAAGGAGGTGATCCAACCGC		
S_chl_GMx_5_F	Forward	CAAGCGCGATGGAAATCTGG	1477	gyrB
S_chl_GMx_5_R	Reverse	TCCGTAATGGATGATGGCCG		
S_chl_GMx_7_F	Forward	ATCGCCGGATTAACGCAGTA	1499	Flagellin fliC
S_chl_GMx_7_R	Reverse	ATACAAACAGCCTGTCGCTG		
S_chl_GMx_8_F	Forward	ACGTAACAGAGACAGCACGTT	1489	Flagelin
S_chl_GMx_8_R	Reverse	GTCGCTGCTGACCCAGAATA		
S_chl_aadS_1_F	Forward	TCCGTTTTGGCTTCTGGTTCT	942	Aminoglycoside resistance protein-aadA2
S_chl_aadS_1_R	Reverse	GCTTAGTGCATCTAACGCCG		
S_chl_int2_F	Forward	GGGTTGGATCCATCAGGCAA	1321	Drug-resistance protein int2
S_chl_int2_R	Reverse	TGCGTACTGATTCCGAGTTCA		
S_der_GnMx_1_F	Forward	TTACGGTGAGAAACCGTGGG	1598	Phase 1 flagellin – fliC
S_der_GnMx_1_R	Reverse	CAATCGCCGGATTAACGCAG		
S_der_GnMx_4_F	Forward	AAACGTATTCGTAAGGATTTTGGT	1833	RNA polymerase beta-subunit- rpoB
S_der_GnMx_4_R	Reverse	CGCCTGAGCGATAACGTAGT		
S_der_aadA_dhfr_F	Forward	CTAACGCTTGAGTTAAGCCGC	1477	aadA1 and dhfr
S_der_aadA_dhfr_R	Reverse	CAACGATGTTACGCAGCAGG		
S_der_int1_F	Forward	GATGCCCGAGGCATAGACTG	1206	Class 1 integron
S_der_int1_R	Reverse	ATGGAGAAGAGGAGCAACGC		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
S_dub_16S_F	Forward	TGATCATGGCTCAGATTGAACG	1559	16S rRNA
S_dub_16S_R	Reverse	GTGAGCACTGCAAAGTACGC		
S_dub_siAB_2_F	Forward	AGTGATCCCCAACTGAAGCG	1185	Cell invasion protein SipC
S_dub_siAB_2_R	Reverse	GGCGTCGATAAGAAAACGGC		
S_dub_aph_ble_F	Forward	ACAGGATGAGGATCGTTTCGC	1259	Kanamycin kinase aph and bleomycin resistance protein ble
S_dub_aph_ble_R	Reverse	CCATCCACGCAGTGACCTC		
S_dub_Blac_R	Forward	TATCCTGGGCCTCATCGTCA	1301	Beta-lactamase bla and
S_dub_Blac_F	Reverse	ACACACGTGGAATTTAGGAAAAACT		
S_ent_16S_F	Forward	GCTCAGATTGAACGCTGGC	1500	16S rRNA
S_ent_16S_R	Reverse	GGTTCCCCTACGGTTACCTTG		
S_ent_sABC_1_F	Forward	AACCAGTGTATTTCACTCTGGAA	1274	Fimbrial protein sefC
S_ent_sABC_1_R	Reverse	CTTCGGTGTTTGTCTGAGCG		
S_ent_sABC_2_F	Forward	CGGTACAAAGCACCGAAGGA	740	Fimbrial subunit
S_ent_sABC_2_R	Reverse	ATCGACTCGTAGATAGCCGC		
S_ent_aadA_F	Forward	TCGCTCCTTGGACAGCTTTT	957	aadA5
S_ent_aadA_R	Reverse	GAACGCCGAGTTAGGCATCA		
S_ent_Blac_2_F	Forward	ATCAGTTTGCCCCAACCACA	1282	Betalactamase – bladha-1
S_ent_Blac_2_R	Reverse	ACACTGATTTCCGCTCTGCT		
S_ent_Blac_4_F	Forward	AGACGTCAGGTGGCACTTTT	1132	Beta-lactamase TEM-1 blaTEM-1
S_ent_Blac_4_R	Reverse	CAGTGGAACGAAAACTCACG		
S_ent_dfrA_qacE_F	Forward	TCCAGAACCTTGACCGAACG	1256	dihydrofolate reductase dfrA25 and quaternary ammonium resistance protein qacEdelta1
S_ent_dfrA_qacE_R	Reverse	TCGAAGAAGGAGTCCTCGGT		
S_ent_estX _sat2_F	Forward	CATCAGCGGGTGACAAAACG	986	Esterase- estX
S_ent_estX _sat2_R	Reverse	CTCACCGGAAAATTGCGAGC		
S_newp_16S_F	Forward	AAACGGTGGCTAATACCGCA	1210	16S rRNA
S_newp_16S_R	Reverse	TCACCGTGGCATTCTGATCC		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
S_new_gyrA_F	Forward	CAATAAACGCCGAGACCACG	1017	Gyrase A – gyrA
S_new_gyrA_R	Reverse	TGCCTTCCACGCGTTTATCT		
S_new_strB_F	Forward	GAGTCCCGTCTGGCAATGAA	1182	Streptomycin resistance protein StrA
S_new_strB_R	Reverse	GATTTGCCGGTGCTTCTGTC		
S_spp_16S_F	Forward	TCCTGGCTCAGATTGAACGC	1458	16S rRNA
S_spp_16S_R	Reverse	ACAAAGTGGTAAGCGCCCTC		
S_spp_fliC_F	Forward	CTGTCGCTGTTGACCCAGAA	1397	Flagellar antigen fliC
S_spp_fliC_R	Reverse	GCGCGAGACATGTTGGAAAC		
S_spp_invA_F	Forward	GCCGGTGAAATTATCGCCAC	1450	Invasion protein invA
S_spp_invA_R	Reverse	TATAACGCGCCATTGCTCCA		
S_typh_16S_F	Forward	ACACATGCAAGTCGAACGGT	1492	16S rRNA
S_typh_16S_R	Reverse	TAAGGAGGTGATCCAACCGC		
S_typh_blT_F	Forward	GCAACCGGGGTCTGACG	1151	Beta-lactamase blaTEM
S_typh_blT_R	Reverse	AGACGTCAGGTGGCACTTTT		
S_typh_Int_1_F	Forward	AGTTATCGGGAATGGCCCTG	1303	Class I 424ntegrin – dfrA1 and aadA1
S_typh_Int_1_R	Reverse	TGGTGATCTCGCCTTTCACG		
S_typh_GMx_1_F	Forward	GGCGCCCCTTATTTCAAACG	848	Aminoglycoside adenyltransferase A aadA1
S_typh_GMx_1_R	Reverse	GCGTCGGCTTGAACGAATTG		
S_typh_qEd_sul_F	Forward	TGAGCTCAGGCGTTAGATGC	915	Multidrug exporter (resistance to quaternary ammonium compound) qacEdelta1 and sulphonamide resistance prot. Sul1deltafusion
S_typh_qEd_sul_R	Reverse	ATTCCTTCACGCTGGCAGAA		
S_typhs_GMx_2_F	Forward	CGCTATCGAGCGTCTGTCTT	1344	Phase 1 flagellin fliC
S_typhs_GMx_2_R	Reverse	GCGCGAGACATGTTGGAAAC		
Y_ent_16S_F	Forward	TGGCTCAGATTGAACGCTGG	1487	16S rRNA
Y_ent_16S_R	Reverse	CCTTGTTACGACTTCACCCCA		
Y_ent_ail_1_F	Forward	AAATGGCTCGATGGGACGTT	1331	Attachment invasion protein ail
Y_ent_ail_1_R	Reverse	ACTTCACTCGGATCAGGGCT		
Y_ent_invA_F	Forward	CTTAATATCGGCGCTGGGGT	1302	Invasin invA
Y_ent_invA_R	Reverse	ACGCGGAACAAAGGTGAGTA		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
Y_ent_yopE_F	Forward	AACAACAAAAACAGCAGCGG	783	yopE
Y_ent_yopE_R	Reverse	GGGGAAACACTACCCCCTTG		
Y_en_y_ORF_ystB_F	Forward	TCTCAACTTTTTGGACACCGC	289	Heat stable enterotoxin type B ystB
Y_en_y_ORF_ystB_R	Reverse	ATGCCTAGCAACCCGCACA		
Y_ent_blaA_F	Forward	TGAAGCACTCTTCGCTACGG	857	Beta-lactamase A
Y_ent_blaA_R	Reverse	GGTCGCAGAGGCCAATACAT		
Crypto_16S_F	Forward	ACCTGACTTTATGGAAGGGTTG	1447	18S rRNA
Crypto_16S_R	Reverse	TTCAATCGGTAGGAGCGACG		
Crypt_COWP_F	Forward	GCCTGAAAAGGCTTGTCCAC	484	Oocyst wall protein COWP
Crypt_COWP_R	Reverse	ACCTGGGGGACATACAGGTT		
Crypt_hsp_F	Forward	TGACTCACAGCGTCAAGCAA		Heat shock protein 70 HSP70
Crypt_hsp_R	Reverse	GGCAGAGGATTGCTCACCAT		
Crypto_p23_F	Forward	GGGTTGTTCATCATCAAAGCCA	440	Sporozoite surface antigen p23
Crypto_p23_R	Reverse	AAAGTATACAAGGGAACTCCCAG		
Cry_ATPbdC_F	Forward	ACAATCAGCAGAGGAGGATGG	1680	ATP binding cassette subfamily C
Cry_ATPbdC_R	Reverse	ACCATAACTCCCTCAATTTCTCCA		
Eim_18S_F	Forward	ACCCACTTAGTGTGGAGTCCT	1244	18S rRNA
Eim_18S_R	Reverse	CGTGCAGCCCAGAACATCTA		
Giar_giard_F	Forward	CTATGTTCACCTCCACCCGT	788	Beta-giardin
Giar_giard_R	Reverse	GCCCTGGATCTTCGAGACG		
Gia_U_IT_L_F	Forward	CTCCCCAAGGACGAAGCCAT	1564	rRNA-small subunit ribosomal RNA and internal transcribed spacer 1 ITS-1
Gia_U_IT_L_R	Reverse	TTCGAGGATCGGTGCTTCTC		
I_suis_18S_1_F	Forward	CCTCAGGAAGGGCAGTGTTT	1534	rRNA-18S ribosomal RNA
I_suis_18S_1_R	Reverse	TTTCCATCTCGGGCACGAAC		
T_suis_18S_2_F	Forward	ATACATGCCTCGAAGCTCGG	1498	rRNA-18S ribosomal RNA
T_suis_18S_2_R	Reverse	CCTGTCCCAGTCACGAGAAC		
B_hst_cytb_F	Forward	AAACAATGCATTCATCGACCTTCC	1074	Bovine Cytochrome b
B_hst_cytb_R	Reverse	ACTGTGCCGGCTGTTGGTATTA		
P_hst_cytb_F	Forward	ACGCATTCATTGACCTCCCA	1080	Porcine Cytochrome b
P_hst_cytb_R	Reverse	GGTTGTTTTCGATGATGCTAGTGA		

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
Bov cor_F_4	Forward	GCTAAGGTCAAAAATACCAAGGTT	1617	Spike
Bov cor_R_4	Reverse	ACCTTGGCCTGTAATACCATAAAGA		
PEDV_F_1	Forward	CTTGAGTGGCTCAACCGAGT	1928	Membrane- Nucleocapsid-envelope
PEDV_R_1	Reverse	GTGGGTACAGCGTTGTTTGC		
PEDV_F_3	Forward	TTATGTTGCCAGCCGTTTGC	1647	Polyprotein
PEDV_R_3	Reverse	ACAGCTCCACCAACATTACA		
TGEV_F_4	Forward	GTGCTGTTGCTGAGCATGAC	1388	ORF1b
TGEV_R_4	Reverse	ACGAGCTCTTGCCTTACCAG		
Bov tor_F_1	Forward	TTGCCCTGAACACCTCATCC	1617	polyprotein 1ab
Bov tor_R_1	Reverse	GAGTTGCAAAACCCACAGCA		
Por tor_F_1	Forward	TGTGGCTACTTGGGTGTTGC	702	Nucleocapsid
Por tor_R_1	Reverse	TTACACAGTGGAGCCAGAGG		
Por cir1_F_1	Forward	CAGCGTCAGTGAAAATGCCA	851	Capsid - replicase
Por cir1_R_1	Reverse	TTGCAAAGTAGTAATCCTCCGA		
Por cir2_F_1	Forward	CACTTCGGCAGCGGCA	543	Capsid - replicase
Por cir2_R_1	Reverse	GTCTTCCAATCACGCTTCTGC		
Bov kob_F_2	Forward	TGCTTCCGAACGGTGTTGAA	1659	Unamed protein product
Bov kob_R_2	Reverse	TGGGTTGGGAGTAGGCAGTA		
Por kob_F_2	Forward	CTGACACCAACCTACAGCGT	803	VP1
Por kob_R_2	Reverse	GTTGAGACCAAAGCGGGAGA		
Bov ent_F_1	Forward	TTTAAAACAGCCTGGGGGTTG	1391	5'UTR
Bov ent_R_1	Reverse	AGGGTCCCCTGGTGGAATTT		
Por ent_F_6	Forward	CTCCCTTCCACACAGAACGG	432	5'UTR
Por ent_R_6	Reverse	AGGGATCCAGTTGCAAAATGAAA		
Por sap_F_1	Forward	GCACCCGACAAGGAAGAAGA	1561	Capsid- VP2
Por sap_R_1	Reverse	CACCAGTTTCAGCTGCTGTC		
Por tes _F_1	Forward	GCTTGGACTCCCCAAGGTTT	1062	VP1
Por tes_R_1	Reverse	CCCAGGGCGTGGACAAAATA		
Bov nor_F_1	Forward	GGACGAACTTGTTGCTCCCA	1085	RdRp
Bov nor_R_1	Reverse	GACCTCAGGAGGGAGGACTT		
Por nor_F_2	Forward	CCACCCACAGTTGAGTCCAA	1577	RdRp-VP2-VP1
Por nor_R_2	Reverse	GGAAAGGGAGGTTGACTGGG		
Neb_F_1	Forward	GGCTAGTGGCTTGGTGGG	1448	RdRp- Capsid
Neb_R_1	Reverse	CAGTCCAAGTGATGCCCGTA		
Sap_F_4	Forward	GCTTGCCCGGATGTCCCTA	1820	Capsid
Sap_R_4	Reverse	CCCTTGCTAGGGGTCACAC		
Bov rotA_F_4	Forward	GGTTCGCTTGCGTACTTGTT	1475	VP4
Bov rotA_R_4	Reverse	ACTAATCCGCATCGTAGTGTT		
Bov rotB_F_1	Forward	TCAGAGATGCCGTTGCCATT	758	VP7

Continued

Primer ID	Forward Reverse	Sequence (5'->3')	Product length (bp)	Gene
Bov rotB_F_1	Forward	TCAGAGATGCCGTTGCCATT	758	VP7
Bov rotB_R_1	Reverse	AGTCCTCTTTATGCTCGCGG		
Bov rotC_F_2	Forward	CGTCAGCTGATATCCCAGAACT	1308	VP4
Bov rotC_R_2	Reverse	TCCTGCTGTCATAAACCAACCA		
Por rotA_F_3	Forward	AAAAGGATGGCCAACAGGGT	521	VP7
Por rotA_R_3	Reverse	CGGTTTTTGGTGCAGTGGTT		
Por rotB_F_1	Forward	TACGCTGCTTCTCGTCCTTG	741	VP7
Por rotB_R_1	Reverse	CTTTATGCTCGCGGTTCTGC		
Por rotC_F_2	Forward	CACCTTATAGCGGACGCACT	882	VP7
Por rotC_R_2	Reverse	AGCCCGATGTCTCGGAGT		
Orthoreo_F_1	Forward	CAGCTACCTCCACTGAGCAC	948	L1
Orthoreo_R_1	Reverse	CCGCTCCATACTTCTCCCAC		
BVDV2_F_1	Forward	CTCGTATACGGATTGGGTGTCA	1225	5'UTR - Npro
BVDV2_R_1	Reverse	CTGTATTCCTTCGGTGCCGT		
BVDV2_F_3	Forward	AGAGTGGTAGCAATGACAGCA	1073	Polyprotein
BVDV2_R_3	Reverse	GTCTGATACCCGACGTAGCC		
Bov par_F_1	Forward	GAGCCTGCCCCAGATATCAC	1021	NSP - Capsid
Bov par_R_1	Reverse	CCATGGGCATTGGACCTTCT		
Por boca_F_1	Forward	TCAGTTCTGGCATGGAGCAA	1264	NS1
Por boca_R_1	Reverse	TGTGTTCTGTTTTCCCGCCT		
Por par_F_1	Forward	CACCCAGGGTCTAAACCACC	1158	VP2
Por par_R_1	Reverse	TGGTCCACCATTGGAGTATTCA		

Appendix V. Probe design software parameters

UPS		Picky		GoArray		eArray	
Probe length	60	Probe length	58-62	Probe length	60	Probe length	60
No probes/seq	5	No probes/seq	5	No probes/seq	1*	No probes/seq	3
Salt concentration	0.58M*	Salt concentration	500mM*	Salt concentration	1*	Salt concentration	-
Probe uniqueness	Pangenomic level Among non-redundant NCBI nucleotide database	Maximum match length	15*	Subsequence length	27	-	
		Minimum match length	10*	Min space between sub-sequences	20*		
		Maximum GC content	70*	Max space between sub-sequences	60*		
		Minimum GC content	30*	Step for specificity search	1*		
		Minimum trigger similarity	75*	Length for random sequence	6		
		Minimum T° difference	15*	Min Tm	83*		
		DNA concentration	1nM*	Max Tm	93*		
Degenerate probes allowed	No*	Screen only forward strands	No*	Max Tm for secondary structures	63*		
		Preferred shared oligo	No*	Folding temperature (0-100°C)	50*		
		Compute Picky oligo using the better salt effect concentration		Database name	NCBI‡		
				Max length for identity	15		
				Ionic conditions [Mg^{++}]	0*		

Appendix V legend

*: By default

‡: BLAST executable were downloaded in January 2014

T°: Temperature

Tm: Melting temperature

Appendix VI. Number of designed probes per pathogen and software – Agilent platform

Pathogen	UPS	Picky	GoArray	eArray	No of probes
Bovine coronavirus	75	39	24	72	210
Porcine epidemic diarrhea virus (PEDV)	115	30	46	117	308
Transmissible gastroenteritis virus (TGEV)	46	45	15	45	151
Bovine torovirus	73	35	29	81	218
Porcine torovirus	97	19	35	105	256
Porcine circovirus (PCV1, PCV2)	119	45	41	108	313
Bovine kobuvirus	45	18	16	42	121
Porcine kobuvirus	87	38	29	87	241
Bovine enterovirus	71	63	27	57	218
Porcine enterovirus	103	85	26	123	337
Sapelovirus	113	35	37	81	266
Teschovirus	118	113	44	129	404
Bovine norovirus	110	59	41	129	339
Porcine norovirus	133	52	44	123	352
Nebovirus	27	19	8	18	72
Sapovirus	93	71	26	72	262
Bovine rotavirus (A, B, C)	278	196	93	225	792
Porcine rotavirus (A, B, C)	250	213	83	300	846
Porcine orthoreovirus	73	55	24	72	224
Bovine parvovirus	20	16	8	9	53
Porcine Bocavirus	87	69	28	78	262
Porcine parvovirus	50	42	21	75	188
Bovine viral diarrhea virus (BVDV1, BVDV2)	261	157	82	243	743
Bacteroides fragilis	174	91	46	120	431
Campylobacter (*coli*, *jejuni*)	469	227	144	378	1218
Clostridium (*difficile, perfringens*)	383	130	123	333	969
Escherichia coli (EPEC, ETEC, STEC, EAEC)	567	310	149	321	1347
Salmonella (Choleraesuis, Derby, Dublin, Enteritidis, Newport, Typhimurium, Typhisuis)	1102	795	313	501	2711
Brachyspira (*hyodysenteriae*, *pilosicoli*)	219	67	93	231	610
Lawsonia intracellularis	86	77	24	33	220

Continued

Pathogen	UPS	Picky	GoArray	eArray	No of probes
Yersinia enterocolitica	125	63	43	99	330
Trichuris suis	41	10	17	51	119
Cryptosporidium spp.	108	56	27	78	269
Giardia spp.	172	17	38	114	341
Eimeria spp.	51	10	13	33	107
Isospora suis	8	10	3	9	30
Host - Bovine	20	1	10	33	64
Host- Porcine	16	1	10	24	51
Total	**5985**	**3379**	**1880**	**4749**	**15993**

Appendix VII. Number of designed probes per pathogen and software – Alere platform

Pathogen	No probes
Bovine coronavirus	11
Porcine epidemic diarrhea virus (PEDV)	9
Transmissible gastroenteritis virus (TGEV)	5
Bovine torovirus	11
Porcine torovirus	18
Porcine circovirus (PCV1, PCV2)	12
Bovine kobuvirus	9
Porcine kobuvirus	7
Bovine enterovirus	5
Porcine enterovirus	11
Porcine sapelovirus	4
Porcine teschovirus	6
Bovine norovirus	10
Porcine norovirus	8
Nebovirus	4
Sapovirus	9
Bovine rotavirus (A, B, C)	18
Porcine rotavirus (A, B, C,)	23
Orthoreovirus	2
Bovine viral diarrhea virus (BVDV2)	9
Bovine parvovirus	3
Porcine bocavirus	4
Porcine parvovirus	3
Total	201

Appendix VIII. Free energies of folding virus probes

Probe name	Delta G	Probe name	Delta G	Probe name	Delta G	Probe name	Delta G	Probe name	Delta G	Probe name	Delta G
Bov cor_1	-1.33	**PEDV 0203**	-0.61	**Por tor_1**	-2.35	**Por cir1_1**	-3.35	**Bov kob 0006**	-1.5	**Por ent_4**	-4.61
Bov cor_2	-0.41	**PEDV 0205**	-0.56	**Por tor_2**	-2.66	**Por cir1_2**	0.46	**Bov kob 0007**	-1.86	**Por ent_5**	-2.06
Bov cor_3	-6.43	**TGEV_1**	-2.47	**Por tor_3**	-1.18	**Por cir1_3**	-3.77	**Bov kob 0008**	-3.76	**Por ent_6**	-2.58
Bov cor_4	-1.72	**TGEV_2**	0.46	**Por tor_4**	-1.71	**Por cir1_4**	-4.15	**Por kob_1**	-8.1	**Por ent_7**	-7.43
Bov cor_5	-1.9	**TGEV 0163**	-0.65	**Por tor_5**	-2.28	**Por cir2_1**	-2.43	**Por kob_2**	-2.89	**Por ent_8**	-4.01
Bov cor_6	-2.61	**TGEV 0166**	-1.69	**Por tor_6**	-1.92	**Por cir2_2**	-5.63	**Por kob_3**	-4.17	**Por ent_9**	-3.27
Bov cor_7	-2.67	**TGEV 0168**	-1.85	**Por tor_7**	0.13	**Por cir2_3**	-1.03	**Por kob_4**	-5.64	**Por ent_10**	-2.1
Bov cor_8	-1.23	**Bov tor_1**	-0.86	**Por tor_8**	-2	**Por cir 0521**	-0.1	**Por kob_5**	-3.24	**Por ent_11**	-4.96
Bov Cor 0227	-6.06	**Bov tor_2**	-7.02	**Por tor_9**	-4.88	**Por cir 0523**	-3.71	**Por kob_6**	1.46	**Por sap_1**	-2.52
Bov Cor 0229	-2.35	**Bov tor_3**	-4.15	**Por tor_10**	-5.21	**Por cir 0525**	-3.23	**Por kob_7**	-2.36	**Por sap_2**	-1.66
Bov Cor 0231	-2.35	**Bov tor 0001**	-2.09	**Por tor_11**	-5.1	**Por cir 0527**	-1.73	**Bov ent_1**	-3.67	**Por sap_3**	-1.57
PEDV_1	-1.69	**Bov tor 0002**	-1.23	**Por tor_12**	-5.51	**Por cir 0529**	-1.04	**Bov ent_2**	-1.92	**Por sap_4**	-6.77
PEDV_2	-3.39	**Bov tor 0003**	0.15	**Por tor_13**	-4.47	**Bov kob_1**	-8.3	**Bov ent_3**	-1.14	**Por tes_1**	-3.45
PEDV_3	-7.16	**Bov tor 0004**	-0.1	**Por tor_14**	-1.39	**Bov kob 0001**	-1.06	**Bov ent_4**	-3.27	**Por tes_2**	-2.11
PEDV_4	-0.15	**Bov tor 0005**	0.46	**Por tor_15**	-0.95	**Bov kob 0002**	-1.65	**Bov ent_5**	-3.71	**Por tes_3**	-1.32
PEDV 0197	-1.95	**Bov tor 0039**	-1.08	**Por tor_16**	-2.08	**Bov kob 0003**	-1.36	**Por ent_1**	-7.86	**Por tes_4**	-0.57
PEDV 0199	-2.89	**Bov tor 0040**	0.3	**Por tor_17**	-1.45	**Bov kob 0004**	-0.7	**Por ent_2**	-5.81	**Por tes_5**	-0.71
PEDV 0201	-0.22	**Bov tor 0041**	-2.35	**Por tor_18**	-3.69	**Bov kob 0005**	-2.98	**Por ent_3**	-2.59	**Por tes_6**	-2.96

Continued

Probe name	Delta G	Probe name	Delta G	Probe name	Delta G	Probe name	Delta G	Probe name	Delta G	Probe name	Delta G
Bov nor_1	-3.97	**Neb_1**	-2.95	**Bov rot A_6**	-0.71	**Por rot A_6**	-0.35	**Orthoreo_1**	-2.28	**Por par_1**	-0.58
Bov nor_2	-4.24	**Neb_2**	-5.7	**Bov rot A_7**	-2.01	**Por rot A_7**	0.36	**Orthoreo_2**	-1.31	**Por par_2**	-0.28
Bov nor_3	-4.7	**Neb_3**	-3.33	**Bov rot A_8**	-3.15	**Por rot A_8**	-4.34	**BVDV 2_1**	-5.54	**Por par_3**	-1.04
Bov nor_4	-2.95	**Neb_4**	-5.51	**Bov rot A_9**	-0.32	**Por rot A_9**	-3.39	**BVDV 2_2**	-7.69		
Bov nor_5	-0.52	**Sap_1**	-1.59	**Bov rot A_10**	-3.81	**Por rot A_10**	-5.73	**BVDV 2_3**	-0.79		
Bov nor_6	-1.21	**Sap_2**	-3.42	**Bovine rotavirus B_1**	-0.89	**Por rot B_1**	-0.8	**BVDV 2_4**	-5.7		
Bov nor_7	0.06	**Sap_3**	-5.59	**Bov rot B_2**	-1.52	**Por rot B_2**	-1.45	**BVDV 2_5**	-1		
Bov nor_8	-2.06	**Sap_4**	-4.43	**Bov rot B_3**	-0.26	**Por rot B_3**	-0.91	**BVDV 0160**	-4.49		
Bov nor_9	-1.09	**Sap_5**	0.03	**Bov rot B_4**	-6.03	**Por rot B_4**	-0.39	**BVDV 0162**	-0.73		
Bov nor_10	-2.64	**Sap_6**	-2.74	**Bov rot B_5**	-3.24	**Por rot B_5**	-1.03	**BVDV 0164**	-2.33		
Por nor_1	-4.73	**Sap_7**	-0.33	**Bov rot C_1**	-3.24	**Por rot C_1**	0.1	**BVDV 0166**	-3.93		
Por nor_2	-1.39	**Sap_8**	-3.8	**Bov rot C_2**	-3.86	**Por rot C_2**	-2.44	**Bov par_1**	-1.74		
Por nor_3	0.22	**Sap_9**	-0.87	**Bov rot C_3**	-0.18	**Por rot C_3**	-0.56	**Bov par_2**	-7.46		
Por nor_4	-4.57	**Bov rot A_1**	-1.98	**Por rot A_1**	-1.9	**Por rot C_4**	-0.89	**Bov par_3**	-1.79		
Por nor_5	-0.75	**Bov rot A_2**	-1.33	**Por rot A_2**	0.04	**Por rot C_5**	-0.82	**Por boca_1**	-0.51		
Por nor_6	-2.49	**Bov rot A_3**	-0.92	**Por rot A_3**	-0.6	**Por rot C_6**	-0.47	**Por boca_2**	-1.22		
Por nor_7	-4.57	**Bov rot A_4**	-1.63	**Por rot A_4**	-4.69	**Por rot C_7**	-1.24	**Por boca_3**	-6.08		
Por nor_8	-4.07	**Bov rot A_5**	-1.73	**Por rot A_5**	-2.27	**Por rot C_8**	-1.22	**Por boca_4**	-1.26		

Appendix IX. Free energies and mismatches of hybridised probes with targets

Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis
Bov_cor_1	-59.2	0	**PEDV 0201**	-59	0	**Bov tor 0040**	-56	0	**Por tor_16**	-7.7	26
Bov_cor_2	-65	0	**PEDV 0203**	-57.8	0	**Bov tor 0041**	-59.1	0	**Por tor_17**	-7.9	25
Bov_cor_3	-58.1	1	**PEDV 0205**	-62.9	0	**Por tor_1**	-47.6	4	**Por tor_18**	-52.2	4
Bov_cor_4	-55.3	2	**TGEV_1**	-62.6	0	**Por tor_2**	-43.9	7	**Por cir1_1**	-67	0
Bov_cor_5	-56.8	2	**TGEV_2**	-48.9	1	**Por tor_3**	-48.9	5	**Por cir1_2**	-61.5	0
Bov_cor_6	-43.5	5	**TGEV 0163**	-59.1	0	**Por tor_4**	-38.1	9	**Por cir1_3**	-66.8	0
Bov_cor_7	-54.6	1	**TGEV 0166**	-53.6	2	**Por tor_5**	-46.4	6	**Por cir1_4**	-70.5	0
Bov_cor_8	-55.5	1	**TGEV 0168**	-52.6	1	**Por tor_6**	-48.1	5	**Por cir2_1**	-69.7	0
Bov Cor 0227	-64.4	0	**Bov tor_1**	-50.9	6	**Por tor_7**	-55.8	1	**Por cir2_2**	-44	15
Bov Cor 0229	-56.1	0	**Bov tor_2**	-46.6	7	**Por tor_8**	-41.5	7	**Por cir2_3**	-68.3	4
Bov Cor 0231	-58	0	**Bov tor_3**	-59	0	**Por tor_9**	-51.2	3	**Por cir 0521**	-57.6	0
PEDV_1	-62.6	0	**Bov tor 0001**	-59	0	**Por tor_10**	-53.5	3	**Por cir 0523**	-66.5	0
PEDV_2	-63.3	2	**Bov tor 0002**	-58.8	0	**Por tor_11**	-49	5	**Por cir 0525**	-69.1	0
PEDV_3	-62.9	0	**Bov tor 0003**	-59.8	0	**Por tor_12**	-29.2	11	**Por cir 0527**	-66.3	0
PEDV_4	-52	0	**Bov tor 0004**	-51.4	0	**Por tor_13**	-41.1	8	**Por cir 0529**	-58.7	1
PEDV 0197	-59.5	0	**Bov tor 0005**	-55.4	0	**Por tor_14**	-35.7	10	**Bov kob_1**	-53.7	7
PEDV 0199	-53.3	0	**Bov tor 0039**	-60.3	0	**Por tor_15**	-51.4	4	**Bov kob 0001**	-59.4	0

Continued

Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis
Bov kob 0002	-62.4	0	**Por ent_3**	-28.7	12	**Bov nor_3**	-47.1	10	**Sap_2**	-9.9	40
Bov kob 0003	-63.6	0	**Por ent_4**	-8.1	38	**Bov nor_4**	-28.1	15	**Sap_3**	-54	5
Bov kob 0004	-58.7	0	**Por ent_5**	-8.9	36	**Bov nor_5**	-8.4	38	**Sap_4**	-23.3	14
Bov kob 0005	-62.9	0	**Por ent_6**	-62.5	0	**Bov nor_6**	-41.8	10	**Sap_5**	-20.5	19
Bov kob 0006	-62.6	0	**Por ent_7**	-55.3	4	**Bov nor_7**	-34.1	12	**Sap_6**	-30	12
Bov kob 0007	-63.4	0	**Por ent_8**	-54.9	5	**Bov nor_8**	-8.8	44	**Sap_7**	-42.6	7
Bov kob 0008	-65.7	0	**Por ent_9**	-46.2	6	**Bov nor_9**	-8.3	38	**Sap_8**	-50.5	7
Por kob_1	-49.5	5	**Por ent_10**	-48.2	5	**Bov nor_10**	-9.4	40	**Sap_9**	-9.1	37
Por kob_2	-59.6	2	**Por ent_11**	-55.5	3	**Por nor_1**	-19.2	21	**Bov rot A_1**	-62	0
Por kob_3	-39.4	9	**Por sap_1**	-39.6	9	**Por nor_2**	-43	9	**Bov rot A_2**	-32.8	12
Por kob_4	-30.7	13	**Por sap_2**	-36.6	9	**Por nor_3**	-10	40	**Bov rot A_3**	-24.7	15
Por kob_5	-34.8	11	**Por sap_3**	-47	5	**Por nor_4**	-10.8	36	**Bov rot A_4**	-20.2	14
Por kob_6	-23.2	14	**Por sap_4**	-22.1	17	**Por nor_5**	-9.9	38	**Bov rot A_5**	-18.9	19
Por kob_7	-27.7	12	**Por tes_1**	-21.2	14	**Por nor_6**	-50.9	8	**Bov rot A_6**	-14.1	20
Bov ent_1	-28.4	17	**Por tes_2**	-7.1	35	**Por nor_7**	-10.8	36	**Bov rot A_7**	-65.3	0
Bov ent_2	-38.6	10	**Por tes_3**	-14	16	**Por nor_8**	-65.6	0	**Bov rot A_8**	-10.6	39
Bov ent_3	-39	14	**Por tes_4**	-61.4	0	**Neb_1**	-56.4	2	**Bov rot A_9**	-12.1	39
Bov ent_4	-26.9	15	**Por tes_5**	-16.7	15	**Neb_2**	-72	0	**Bov rot A_10**	-15.3	29

Continued

Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis	Probe ID	DeltaG kcal/ mole	No of mis
Bov rot A_2	-32.8	12	**Bov rot C_3**	-56.1	2	**Por rot C_1**	-21.9	19	**BVDV 0162**	-60.5	0
Bov rot A_3	-24.7	15	**Por rot A_1**	-31.5	12	**Por rot C_2**	-62.8	0	**BVDV 0164**	-56.6	0
Bov rot A_4	-20.2	14	**Por rot A_2**	-35.4	13	**Por rot C_3**	-10.2	16	**BVDV 0166**	-60.7	0
Bov rot A_5	-18.9	19	**Por rot A_3**	-15.2	22	**Por rot C_4**	-27.9	14	**Bov par_1**	-11.6	40
Bov rot A_6	-14.1	20	**Por rot A_4**	-36	9	**Por rot C_5**	-63	0	**Bov par_2**	-12.9	36
Bov rot A_7	-65.3	0	**Por rot A_5**	-9.3	40	**Por rot C_6**	-8.2	34	**Bov par_3**	-68.2	0
Bov rot A_8	-10.6	39	**Por rot A_6**	-19.4	16	**Por rot C_7**	-21.6	15	**Por boca_1**	-54.3	1
Bov rot A_9	-12.1	39	**Por rot A_7**	-52.3	3	**Por rot C_8**	-21.3	17	**Por boca_2**	-25.6	13
Bov rot A_10	-15.3	29	**Por rot A_8**	-13.5	21	**Orthoreo_1**	-54.6	5	**Por boca_3**	-62.1	3
Bovine rotavirus B_1	-30	11	**Por rot A_9**	-25.2	13	**Orthoreo_2**	-29.7	14	**Por boca_4**	-8.5	38
Bov rot B_2	-56	0	**Por rot A_10**	-39.3	8	**BVDV 2_1**	-25	16	**Por par_1**	-61.2	0
Bov rot B_3	-32.4	11	**Por rot B_1**	-28.8	9	**BVDV 2_2**	-34	9	**Por par_2**	-58.5	0
Bov rot B_4	-11.1	36	**Por rot B_2**	-9.9	39	**BVDV 2_3**	-9.8	37	**Por par_3**	-59	1
Bov rot B_5	-63.9	0	**Por rot B_3**	-41.2	8	**BVDV 2_4**	-67	1			
Bov rot C_1	-60.1	1	**Por rot B_4**	-35.4	11	**BVDV 2_5**	-25.5	15			
Bov rot C_2	-59.5	3	**Por rot B_5**	-45	10	**BVDV 0160**	-60.4	0			

Appendix X. Detected pathogens with published primers in bovine samples

Sample	Detected pathogens							
2bis	E.coli/Parvo	-						
6?	E.coli/Parvo							
11	E.coli/Parvo	Rota/BVD						
12		Rota/BVD						
13	E.coli/Parvo		Noro/Campy					
14	E.coli/Parvo							
26								
27	E.coli/Parvo							
37	E.coli/Parvo							
58	E.coli/Parvo							
59								
60								
63								
68	NP	NP	NP	NP	NP	NP	NP	NP
1								
3	E.coli/Parvo			Yer/Corona				
5	E.coli/Parvo							
6	E.coli/Parvo			Yer/Corona				
7	E.coli/Parvo	Rota/BVD		Yer/Corona				
8	E.coli/Parvo			Yer/Corona				
9				Yer/Corona				
10	E.coli/Parvo							

Continued

Sample	Detected pathogens							
11								
12	E.coli/Parvo	Rota/BVD			Toro/Campy			
13				Yer/Corona				
14	E.coli/Parvo	Rota/BVD			Toro/Campy			
B1	E.coli/Parvo/Calici			Entero/Corona/Bact		Crypto	Yersinia	
Hem								Kobu
1/3	E.coli/Parvo/Calici	Rota/BVD					Yersinia	Kobu
5/7	E.coli/Parvo/Calici	Rota/BVD						
2/8	E.coli/Parvo/Calici						Yersinia	Kobu
3/10	E.coli/Parvo/Calici							
6/5								
4J								
09		Rota/BVD						
12								
12'		Rota/BVD						
161	E.coli/Parvo/Calici							
162	E.coli/Parvo/Calici	Rota/BVD						
165								
19.1	E.coli/Parvo/Calici	Rota/BVD						
19.2	E.coli/Parvo/Calici	Rota/BVD		Entero/Corona/Yer				
19.3	E.coli/Parvo/Calici	Rota/BVD		Entero/Corona/Yer				
3T								
4T								

Continued

Sample	Detected pathogens							
6T	E.coli/Parvo			Entero/Corona/Yer				
7T								
9T	E.coli/Parvo							
13T								
18T								
20T	E.coli/Parvo							
21T	E.coli/Parvo							
25T								
29T								
30T	E.coli/Parvo							
31T	E.coli/Parvo							
35T	E.coli/Parvo							
38T								
40T								
41T								
42T								
43T								
45T				Entero/Corona/Yer				
S1								
S2	E.coli/Parvo			Entero/Corona/Yer				
S3	E.coli/Parvo			Entero/Corona/Yer				
S4								
S5	E.coli/Parvo							

Continued

Sample	Detected pathogens							
S6	E.coli/Parvo							
1	E.coli/Parvo							
2	E.coli/Parvo							
3				Entero/Corona/Yer				
4	E.coli/Parvo							
5								
6								
A								
B2								
C								
D								
E								
F	E.coli/Parvo							
G				Entero/Corona/Yer				
C1								
C2	E.coli/Parvo							
C3								
C4								
C5	E.coli/Parvo							
C6								
C7	E.coli/Parvo							
C8	E.coli/Parvo							
C10								Kobu

Continued

Sample	Detected pathogens							
C11								
C12								
C13								
C14								
C15								
C16								
C17								
C18								
Va								
Sva								
V1								
V2								
Ve1								
Ve2								
32								
94								
15								
Cam1	E.coli/Parvo							
Cam2	E.coli/Parvo							
Cam3	E.coli/Parvo							
Cam4	E.coli/Parvo							
Cam5	E.coli/Parvo							
cap								

Continued

Sample	Detected pathogens							
44								
47								
49								
53	E.coli/Parvo							
54								
55	E.coli/Parvo							
Tl1	E.coli/Parvo							
Tl2	E.coli/Parvo							
Tl3	E.coli/Parvo							
Tl4	E.coli/Parvo							
Tl5	E.coli/Parvo							
Tl6	E.coli/Parvo							
Tl7	E.coli/Parvo							
Tl8	E.coli/Parvo							

E.coli : *Escherichia coli*, Entero: Enterovirus, Parvo: Bovine parvovirus , Bact: *Bacteroides fragilis*, Yer: *Yersinia,* Calici: Calicivirus, Corona : Coronavirus, Crypto: *Cryptosporidium*, Rota : Rotavirus A, Toro : Torovirus, BVD: BVDV, Kobu: Kobuvirus, Noro : Norovirus, Campy : Campylobacter, NP: Not performed.

Appendix XI. Detected pathogens with designed primers in bovine samples

Sample	Designed primers									
	Multiplex			Sample	Monoplex					
2bis				**2bis**						
6?				**6?**						
11				**11**						
12				**12**						
13	Corona/kobu/EPEC eae	Crypto/Rota/Entero		**13**	Salm					
14				**14**						
26				**26**						
27	Corona/kobu/EPEC eae			**27**	Salm					
37	Corona/kobu/EPEC eae			**37**						
58	Corona/kobu/EPEC eae	Crypto/Rota/Entero		**58**	Salm					
59				**59**						
60				**60**						
63				**63**						
68				**68**						
1				**1**	Salm					
3	Corona/EPEC eae		Kobu	**3**	Salm					
5				**5**	Salm					
6				**6**						
7	Corona/EPEC eae			**7**	Salm					
8				**8**						
9				**9**						

Continued

Sample	Designed primers									
	Multiplex			Sample	Monoplex					
10	Corona/EPEC eae			**10**	Salm	E.coli F5	Crypto			
11				**11**						
12	Corona/EPEC eae			**12**	Salm					
13				**13**						
14	Corona/EPEC eae		Kobu	**14**	Salm	E.coli F5				
B1	Corona/EPEC eae			**B1**	Salm					
B2	Corona/EPEC eae			**B2**	Salm					
Hem				**Hem**						
1/3	Corona/EPEC eae			**1/3**	Salm					
5/7	Corona/EPEC eae			**5/7**	Salm					
2/8				**2/8**						
3/10	Corona/EPEC eae			**3/10**	Salm					
6/5				**6/5**						
4J				**4J**						
09	Corona/EPEC eae			**09**	Salm		Crypto			
12				**12**						
12'	Corona/EPEC eae			**12'**	Salm					
161	Corona/EPEC eae			**161**	Salm	E.coli F5				
162	Corona/EPEC eae			**162**	Salm					
165				**165**						
19.1	Corona/EPEC eae			**19.1**	Salm		Crypto			
19.2	Corona/EPEC eae			**19.2**	Salm		Crypto			

Continued

Sample	Designed primers									
	Multiplex			Sample	Monoplex					
19.3	Corona/EPEC eae			**19.3**	Salm		Crypto			
3T				**3T**	Salm					
4T				**4T**	Salm					
6T	Corona/EPEC eae			**6T**	Salm					
7T				**7T**	Salm					
9T	Corona/EPEC eae			**9T**	Salm					
13T				**13T**	Salm					
18T				**18T**	Salm					
20T	Corona/EPEC eae			**20T**	Salm					
21T	Corona/EPEC eae			**21T**	Salm					
25T				**25T**	Salm			BVD		
29T				**29T**	Salm		Crypto			
30T				**30T**	Salm					
31T				**31T**	Salm					
35T	Corona/EPEC eae			**35T**	Salm					
38T				**38T**	Salm					
40T				**40T**	Salm					
41T				**41T**	Salm					
42T				**42T**	Salm					
43T				**43T**	Salm					
45T	Corona/EPEC eae			**45T**	Salm					
S1				**S1**						

Continued

Sample	Designed primers									
	Multiplex			Sample	Monoplex					
S2				**S2**						
S3	Corona/EPEC eae			**S3**	Salm		Crypto		E.coli eae	
S4				**S4**						
S5	Corona/EPEC eae			**S5**	Salm					
S6	Corona/EPEC eae			**S6**	Salm					
1	Corona/EPEC eae	Crypto/Salm/Entero		**1**	Salm					
2	Corona/EPEC eae	Crypto/Salm/Entero		**2**	Salm					
3				**3**						
4	Corona/EPEC eae			**4**	Salm					
5				**5**						
6	Corona/EPEC eae			**6**	Salm		Crypto			
A				**A**						
B2				**B2**						
C				**C**						
D				**D**						
E	Corona/EPEC eae			**E**	Salm			BVD		
F	Corona/EPEC eae			**F**	Salm				E.coli eae	
G				**G**						
C1				**C1**						
C2				**C2**	Salm			BVD		
C3	Corona/EPEC eae			**C3**	Salm					

Continued

Sample	Designed primers									
	Multiplex			Sample	Monoplex					
C4	Corona/EPEC eae			**C4**	Salm	E.coli F5		BVD		
C5	Corona/EPEC eae			**C5**	Salm		Crypto			
C6				**C6**						
C7	Corona/EPEC eae			**C7**	Salm					
C8	Corona/EPEC eae			**C8**						
C10				**C10**						
C11	Corona/EPEC eae			**C11**	Salm		Crypto			
C12	Corona/EPEC eae			**C12**	Salm					
C13	Corona/EPEC eae			**C13**	Salm					
C14	Corona/EPEC eae			**C14**	Salm					
C15	Corona/EPEC eae			**C15**	Salm			BVD		
C16				**C16**	Salm					
C17				**C17**						
C18	Corona/EPEC eae			**C18**	Salm					
Va	Corona/EPEC eae			**Va**	Salm					
Sva	Corona/EPEC eae			**Sva**	Salm					
V1				**V1**	Salm					Kobu
V2	Corona/EPEC eae			**V2**	Salm					Kobu
Ve1	Corona/EPEC eae			**Ve1**	Salm					
Ve2	Corona/EPEC eae			**Ve2**	Salm					
32	Corona/EPEC eae			**32**	Salm					
94	Corona/EPEC eae			**94**	Salm					

Continued

Sample	Designed primers									
	Multiplex			Sample	Monoplex					
15	Corona/EPEC eae			**15**	Salm					
Cam1	Corona/EPEC eae		Kobu	**Cam1**	Salm	E.coli F5				
Cam2	Corona/EPEC eae		Kobu	**Cam2**	Salm	E.coli F5				
Cam3	Corona/EPEC eae		Kobu	**Cam3**	Salm	E.coli F5				
Cam4	Corona/EPEC eae		Kobu	**Cam4**	Salm	E.coli F5				
Cam5	Corona/EPEC eae		Kobu	**Cam5**	Salm					
cap	Corona/EPEC eae			**cap**	Salm					
44	Corona/EPEC eae			**44**	Salm					
47	Corona/EPEC eae			**47**	Salm					
49				**49**	Salm					
53				**53**	Salm					
54				**54**	Salm					
55	Corona/EPEC eae	Salm/Entero		**55**	Salm	E.coli F5				
Tl1	Corona/EPEC eae			**Tl1**	Salm	E.coli F5	Crypto			
Tl2	Corona/EPEC eae			**Tl2**	Salm	E.coli F5	Crypto			
Tl3	Corona/EPEC eae			**Tl3**	Salm	E.coli F5	Crypto			
Tl4	Corona/EPEC eae			**Tl4**	Salm	E.coli F5	Crypto			
Tl5	Corona/EPEC eae			**Tl5**	Salm	E.coli F5	Crypto			
Tl6	Corona/EPEC eae			**Tl6**	Salm	E.coli F5	Crypto			
Tl7	Corona/EPEC eae			**Tl7**	Salm	E.coli F5	Crypto			
Tl8	Corona/EPEC eae			**Tl8**	Salm	E.coli F5	Crypto			

Appendix XI legend

E. coli: *Escherichia coli*, Entero: Enterovirus, Campy: *Campylobacter,* Parvo: Bovine parvovirus, Bact: *Bacteroides fragilis,* Yer : *Yersinia,* Calici: Calicivirus, Corona: Coronavirus, Crypto: *Cryptosporidium,* Rota : Rotavirus A, Toro: Torovirus, BVD: BVDV, Kobu: Kobuvirus, Noro: Norovirus, NP: Not performed.

Appendix XII. Detected P. kobuvirus and *C. difficile* by monoplex PCR in porcine samples (Designed primers)

Porcine kobuvirus				*Clostridium difficile*				
Designed Monoplex				Designed Monoplex				Elisa Results
Batch No	Sample ID	Lab sample ID	VP 1	Batch No	Sample ID	Lab sample ID	16S rRNA	
Batch 1	PM1B278	1	NP	Batch 1	PM1B278	1	-	-
	PM2A3	2	NP		PM2A3	2	-	-
	PM2A1	3	NP		PM2A1	3	-	-
	PM2B269	4	-		PM2B269	4	+	+
	PM2A2	5	NP		PM2A2	5	-	-
	PM2B266	6	-		PM2B266	6	+	+
	PM2B262	7	NP		PM2B262	7	-	-
	PM3B1	8	NP		PM3B1	8	-	-
	PM4A221	9	-		PM4A221	9	-	+
Batch 2	PM2B271	1	-	Batch 2	PM2B271	1	-	+
	PM4A231	2	-		PM4A231	2	NP	+
	PM4B472	3	-		PM4B472	3	NP	+
	PM5A416	4	-		PM5A416	4	+	+
	PM8B3	5	-		PM8B3	5	-	-
	PM8B48	6	-		PM8B48	6	+	+
	PM17A416	7	-		PM17A416	7	+	-
	PM17A418	8	+		PM17A418	8	+	+
	PM17A420	9	-		PM17A420	9	+	+
	PM17A421	10	-		PM17A421	10	+	+
	PM17A428	11	-		PM17A428	11	+	+
	PM18A447	12	-		PM18A447	12	+	+
	PM18A451	13	+		PM18A451	13	+	+
	PM21B157	14	-		PM21B157	14	+	+
	PM23A392	15	-		PM23A392	15	+	+
Batch 3	PM8A469	8A	-	Batch 3	PM8A469	8A	+	+
	PM8B44	8B	-		PM8B44	8B	+	+
	PM9A495	9	+		PM9A495	9	+	+
	PM9A497	9'	-		PM9A497	9'	+	+
	PM13A468	13	-		PM13A468	13	+	+
	PM15B69	15	-		PM15B69	15	+	+
	PM16A128	16	-		PM16A128	16	+	+

Continued

Porcine kobuvirus				*Clostridium difficile*				
Designed Monoplex				Designed Monoplex				Elisa Results
Batch No	Sample ID	Lab sample ID	VP1	Batch No	Sample ID	Lab sample ID	16S rRNA	
Batch 4	PM4B77	A	+	Batch 4	PM4B77	A	+	+
	PM5A421	B	-		PM5A421	B	+	+
	PM16A137	C	+		PM16A137	C	+	+
	PM17B167	D	+		PM17B167	D	-	+
	PM21A500	E	-		PM21A500	E	+	+
	PM22A216	F	+		PM22A216	F	+	+
	PM23A398	G	+		PM23A398	G	+	+
	PM23A407	H	-		PM23A407	H	+	+
	PM24A237	I	+		PM24A237	I	+	+
Batch 5	PM5A417	5A	-	Batch 5	PM5A417	5A	-	-
	PM9A1	9A	-		PM9A1	9A	-	-
	PM15A444	15A	-		PM15A444	15A	+	-
	PM15B1	15B	-		PM15B1	15B	-	-
	PM17A422	17A	-		PM17A422	17A	-	-
Batch 6	PM8A2	1B	-	Batch 6	PM8A2	1B	NP	-
	PM18A450	2B	+		PM18A450	2B	NP	-
	PM8A3	4B	+		PM8A3	4B	NP	-
	PMB2271	5B	-		PMB2271	5B	NP	+
	PM1A1	6B	-		PM1A1	6B	NP	-
	-	7B	+		-	7B	NP	-

Appendix XIII. Detected *C. difficile* and toxins, P. kobuvirus and P. rotavirus A by multiplex PCR in porcine samples (Designed primers)

Batch No	Sample ID	Lab sample ID	*C.diff* 16S rRNA	*tcdA*	*tcdB*	P. rotavirus A	P. kobuvirus
Batch 1	PM1B278	1	NP	NP	NP	NP	NP
	PM2A3	2	NP	NP	NP	NP	NP
	PM2A1	3	NP	NP	NP	NP	NP
	PM2B269	4	-	-	-	-	-
	PM2A2	5	NP	NP	NP	NP	NP
	PM2B266	6	-	-	-	-	-
	PM2B262	7	NP	NP	NP	NP	NP
	PM3B1	8	NP	NP	NP	NP	NP
	PM4A221	9	-	-	-	-	-
Batch 2	PM2B271	1	NP	NP	NP	NP	NP
	PM4A231	2	NP	NP	NP	NP	NP
	PM4B472	3	NP	NP	NP	NP	NP
	PM5A416	4	NP	NP	NP	NP	NP
	PM8B3	5	NP	NP	NP	NP	NP
	PM8B48	6	-	-	-	-	-
	PM17A416	7	-	-	-	+	-
	PM17A418	8	+	-	+	-	-
	PM17A420	9	+	-	-	-	-
	PM17A421	10	+	-	-	-	-
	PM17A428	11	+	-	-	-	-
	PM18A447	12	+	-	-	-	-
	PM18A451	13	-	-	-	-	-
	PM21B157	14	+	-	+	-	-
	PM23A392	15	+	-	+	+	-
Batch 3	PM8A469	8A	-	-	-	-	-
	PM8B44	8B	-	-	-	-	-
	PM9A495	9A	+	+	+	-	+
	PM9A497	9A'	+	-	-	-	-
	PM13A468	13	+	-	+	-	-
	PM15B69	15	+	-	+	-	-
	PM16A128	16	+	-	+	-	-
Batch 4	PM4B77	A	-	-	-	-	-
	PM5A421	B	-	-	-	-	-
	PM16A137	C	+	-	+	+	-

Continued

Batch No	Sample ID	Lab sample ID	*C.diff* 16S rRNA	*tcdA*	*tcdB*	P. rotavirus A	P. kobuvirus
Batch 4	PM17B167	D	-	-	-	-	+
	PM21A500	E	+	-	-	-	-
	PM22A216	F	-	-	-	-	-
	PM23A398	G	+	-	+	+	+
	PM23A407	H	-	-	-	-	-
	PM24A237	I	+	-	-	+	+
Batch 5	PM5A417	5A	-	-	-	+	-
	PM9A1	9A	-	-	-	-	-
	PM15A444	15A	-	-	-	+	-
	PM15B1	15B	-	-	-	+	-
	PM17A422	17A	-	-	-	+	-
Batch 6	PM8A2	1B	-	-	-	-	-
	PM18A450	2B	-	-	-	-	-
	PM8A3	4B	-	-	-	-	-
	PMB2271	5B	-	-	-	-	-
	PM1A1	6B	-	-	-	-	-
	-	7B	-	-	-	-	+

C.diff 16S rRNA: *C. difficile* 16S rRNA, *tcdA*: *Clostridium difficile* toxin A, *tcdB*: *Clostridium difficile* toxin B, P. Rotavirus A: Porcine rotavirus A, P. kobuvirus: Porcine kobuvirus. +: Presence, -: Absence

Appendix XIV. Signal intensities of samples 7, 8, 13 and 14 subjected independently to random amplification followed by biotin labelling

Probe	Sample 7	Sample 8	Sample 13	Sample 14
Biotin-Marker_2,5µM	**0.777516**	**0.58222**	**0.539063**	**0.703152**
PEDV_2	0.512336	0.029444	-0.00519	0.670467
PEDV_3	0.651884	0.030987	0.002217	0.584162
PEDV_4	0.553188	0.005721	0.003182	0.339779
PEDV 0205	0.625129	0.204608	0.236088	0.123653
TGEV_1	0.58269	0.662393	0.565891	0.75443
TGEV_2	0.744684	0.240547	0.567729	0.597346
TGEV 0163	0.005532	0.364991	0.003432	0.023453
TGEV 0166	0.808646	0.250895	0.90033	0.5587
TGEV 0168	0.463134	0.588739	0.551669	0.595406
Bov tor_2	0.001743	0.422587	0.004313	0.041721
Por cir1_1	0.004643	0.438041	0.005579	0.049717
Por cir2_3	0.32647	0.55522	0.563785	0.477116
Por sap_2	0.241373	0.431514	0.233712	0.403965
Biotin-Marker_2,5µM	**0.751347**	**0.604388**	**0.56949**	**0.703964**
Por nor_6	0.191531	0.370048	0.404596	0.416366
Por rot A_2	0.065285	0.000901	0.005522	0.529528
Por rot A_3	0.45605	0.52998	0.579532	0.746426
Biotin-Marker_2,5µM	**0.766016**	**0.535529**	**0.592841**	**0.712436**

Appendix XV. Signal intensities of samples 7, 8, 13 and 14 subjected independently to sequence-specific amplifications followed by biotin labelling

Probe	Sample 7	Sample 8	Sample 13	Sample 14
Biotin-Marker_2,5µM	**0.25523**	**0.605423**	**0.70478**	**0.750074**
Por tor_17	0.412285	0.010308	0.004957	0.001956
Bov kob 0007	0.058732	0.752405	0.786338	0.001867
Bov kob 0008	0.533254	0.377577	0.089959	0.00739
Por kob_1	0.004536	0.217717	0.446591	0.019242
Por kob_2	0.004653	0.813343	0.781785	0.006831
Por kob_3	-0.002622	0.336804	0.050383	0.05531
Por kob_4	0.829349	0.817955	0.817275	0.008906
Por kob_5	0.814598	0.737643	0.804912	0.001838
Por kob_6	0.671922	0.725688	0.731479	0.057226
Por kob_7	0.446721	0.802566	0.801338	0.004725
Por ent_9	-0.003159	0.619681	0.013805	0.000006
Por ent_10	0.514808	0.008306	0.006267	0.005633
Biotin-Marker_2,5µM	**0.288307**	**0.588566**	**0.682867**	**0.727981**
Bov rot A_8	-0.000651	0.400272	0.010227	-0.003261
Por rot A_3	0.505123	0.501735	0.012585	0.759454
Por rot A_4	0.048656	0.501292	0.002088	0.002934
Por rot B_5	0.032838	0.424105	0.005609	0.006421
Por rot C_1	0.003328	0.469986	0.001097	0.010394
BVDV 2_2	0.004713	0.56514	0.00617	0.050906
BVDV 0160	0.002155	0.00999	0.001932	0.460424
Por boca_4	0.026241	0.477027	0.00209	0.00461
Por par_3	0.008684	-0.004357	0.00411	0.534588
Biotin-Marker_2,5µM	**0.164145**	**0.623102**	**0.717679**	**0.781124**