Detection and quantification of airborne viral and bacterial pathogens associated with bovine respiratory disease complex

By

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Abstract

Airborne pathogens are considered to be sources of respiratory disease infection in calf barns. Different types and quantities of airborne pathogens are present in calf barns and are associated with bovine respiratory disease complex (BRD). In order to detect these airborne pathogens, Oxoid and MD8 air samplers were used inside of a barn for collecting air samples. However, air sampler devices, sampling volume, and sampling duration remain unclear for collecting airborne pathogens from calf barns. Therefore, this study aimed to detect and quantify airborne viral and bacterial pathogens associated with BRD complex. The pilot study aimed to determine the optimum conditions for the use of air samplers, as well as to isolate total airborne bacteria inside of the calf barn and determine colony-forming unit (CFU) counts using an Oxoid air sampler. Furthermore, the longitudinal study aimed to collect nucleic acid from total airborne bacteria and viruses using an MD8 sampler to allow the detection and quantification of RNA for parainfluenza 3 virus (PI3) and bovine respiratory syncytial virus (BRSV), and of DNA for bovine herpesvirus 1 (BoHV-1), and the total bacteria through the use of qPCR assays. The pilot study results showed that the optimal air volumes using an Oxoid air sampler on blood agar plates (BA) and eosin methylene blue plates (EMB) for collecting the total bacteria inside of the barn were 10 and 25 litres, respectively. These air volumes were relatively consistent with the low variance in microbial counts in replicate samples. Similarly, the volume for collecting air samples on gelatine filters using an MD8 sampler was 800 litres, which was chosen to shorten the sampling time so as not to disturb the calves. The results from the longitudinal study for microbial counts showed different microbial numbers inside of the barn, and the CFU of the gram-positive bacteria (18,219 ± 11,676 (SD) CFU/m^3) was higher than

that of the gram-negative bacteria (2,013 ± 1,111 (SD) CFU/m^3). Both bacteria were not affected by barn factors such as temperature, humidity, and the number of calves. Additionally, we found that the younger calves below the age of six weeks were more susceptible to BRD than were those above the age of six weeks.

Moreover, the detection and quantification of DNA and RNA nucleic acid showed that two RNA viruses, i.e. PI3 and BRSV, were consistently detected in air samples inside of the calf barn, with the viral load ranging from 408 to 70 and from 0.36 to 0.015 median tissue culture infectious dose ($TCID_{50}$) equivalent copies/33 litres of air, respectively, while BoHV-1 was negative during the study. Due to the farm carrying out vaccination schemes against PI3 and BRSV, but not against BoHV-1, it was not possible to find out whether these types of strains originated from the given vaccines or from infection. Therefore, further investigation, such as using viral sequencing to differentiate between the field and vaccine strains, should be considered.

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Author's declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Nottingham.

The work is original and has not been submitted for any other degree at the University of Nottingham or elsewhere.

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List of Abbreviations

BA	Blood Agar plate
BoCV	Bovine Coronavirus
BoHV-1	Bovine Herpesvirus -1
BRD	Bovine Respiratory Disease
BRSV	Bovine Respiratory Syncytial Virus
BVDV	Bovine Viral Diarrhoea Virus
cDNA	Complementary Deoxyribonucleic Acid
CFU/m^3	Colony-Forming Units per cubic metre
°C	Degree Celsius
DNA	Deoxyribonucleic Acid
dsRNA	Double Stranded Ribonucleic Acid
E	Early
EMP	Eosin methylene blue
F	Fusion protein
°F	Degree Fahrenheit
G	Glycoprotein
H. somni	*Histophilus Somni*
IBR	Infectious Bovine Rhinotracheitis virus
IE	Immediate-early
Kb	Kilobase
L	Litre
L protein	Polymerase protein
M	Matrix protein

TCID50	Median Tissue Culture Infectious Dose
M	Metre
M. haemolytica	*Mannheimia Haemolytica*
Min	Minute
N	Nucleoprotein
P	Phosphoprotein
P. multocida	*Pasteurella multocida*
PI	persistent infections
PCR	Polymerase Chain Reaction
PI3	Parainfluenza 3 Virus
PPTV	Positive Pressure Tube Ventilation
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT- PCR	Real-Time Polymerase Chain Reaction
Sec	Second
SH	Small hydrophobic protein
ssRNA	Single stranded RNA
UK	United Kingdom
US	United State
Wk	Week
WSD	Wisconsin healthy scoring system

Chapter 1: Introduction

1.1 An overview of the bovine respiratory disease complex

Bovine respiratory disease complex (BRD), also commonly known as shipping fever in cattle and enzootic calf pneumonia in young beef and dairy calves, is an ambiguous expression used to represent an infectious pneumonia complex (Callan and Garry 2002, Dane et al. 2019, Lillie 1974). BRD can encompass a range of respiratory illnesses, from those that are acute and fatal to chronic, prolonged, and intractable pneumonic disease (Klima et al. 2014). BRD affects the lower and upper respiratory system, leading to mild or severe inflammation. Depending on the exposure to infection in different tissues the disease can be associated with bronchitis, tracheitis, rhinitis and pharyngitis prompting the development of pneumonia (Gershwin et al. 2015). BRD can cause substantial morbidity and mortality, due to acute pneumonia, in neonatal, weaned, and growing calves. Furthermore, in the chronic form, the disease leads to weakness, poor performance and welfare concerns, as illness often requires early culling of affected animals (Gershwin et al. 2015; Urban-Chmiel and Grooms 2012).

BRD is regarded as one of the most widespread diseases affecting cattle worldwide (Griffin 1997; Urban-Chmiel and Grooms 2012; Gershwin et al. 2015) with devastating economic implications to the UK cattle industry, costing the United Kingdom more than £80 million per annum (NADIS, 2020). BRD affects approximately 1.9 million cattle in the UK annually (Nicholas 2011; Nicholas and Ayling 2003). A recent study in Ireland showed that 10% of calf mortalities were attributed to BRD, and in the United Kingdom, a BRD prevalence of 45.9% and incidence of 10.1% was found (Johnson and Pendell 2017). Australia has reported a BRD incidence of 18.2% medium in large farms

(Hay et al. 2014). The mortality rate of Danish dairy calves in 2014 was estimated to be 7–10%, and BRD was considered responsible for at least 10–35% of deaths (Grønbæk et al., 2016). In addition, more than 90% of cattle in the United States are affected by BRD after the entry of cattle to the new herds (Hay et al. 2014).

These high mortality and morbidity rates may accrue due to the production systems used on the farm. There are two housing systems commonly used in livestock: group housing or housed in individual pens (Teagasc | Agriculture and Food Development Authority, 2022). US farms often use group housing where a group of 80 - 100 calves are housed together. European countries, including the UK, are using both housing systems, but they have frequently used individual pens for calves. A study by Curtis et al. (2016) in the UK, comparing the group housing and housed in individual pen showed that the calves in group housing are more susceptible to diseases such as diarrhoea and pneumonia compared with calves in individual pens. This is because of the air temperature, humidity, ventilation, concentrations of noxious gases, and dust particles due to the calves' high stocking density (Curtis et al. 2016).

The respiratory tract of the calves consists of upper and lower parts; the upper respiratory tract includes the nose, pharynx, and larynx. The lower respiratory tract consists of the trachea, bronchial tree, and lungs (Veit and Farrell 1978). These tracts open to the nose which in turn opens to the outside environment and are lined with mucous membranes. The bovine lung has a relatively high degree of anatomical compartmentalization relative to other species (Veit and Farrell 1978). This compartmentalization can predispose to airway hypoxia or anoxia distal to airways that become occluded. Furthermore, the bovine has low numbers of alveolar macrophages which usually are found in alveolar lumen or airways. These

cells are vital to normal pulmonary clearance, as such, their scarcity in the bovine lung air space may relate to predisposition of cattle to develop respiratory disease (Veit and Farrell 1978).

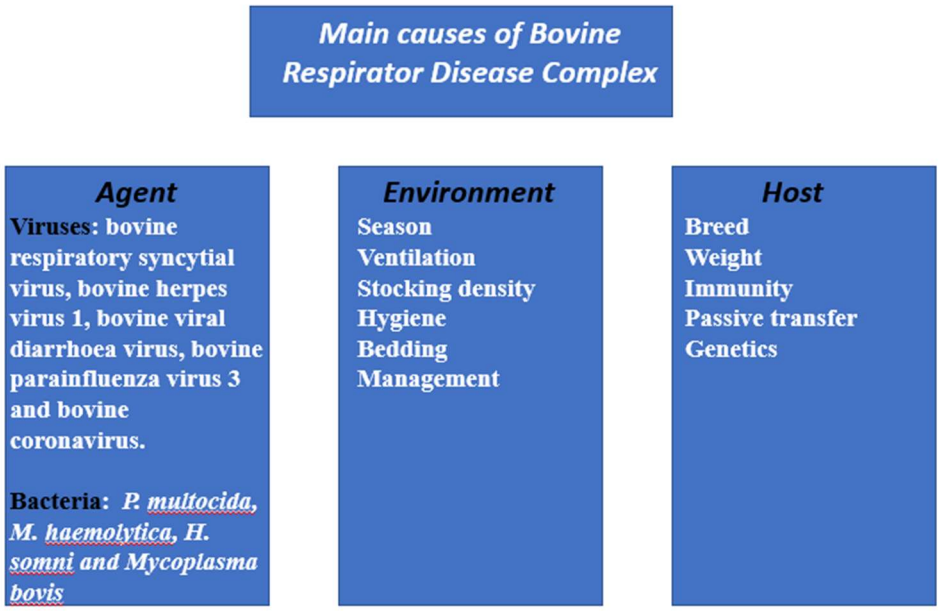

Figure 1.1: The common causes of bovine respiratory disease complex. Modified figure from FAAST (2021).

The ubiquity of the disease is attributed to the interplay of pathogenic agents as normal inhabitants in healthy calves with environmental, physical, genetic, and microbial factors (Snowder 2009). The severity and clinical extent of BRD depend on the exposure to environmental factors and microbial pathogens (viruses and bacteria) (Urban-Chmiel and Grooms 2012; Gershwin et al. 2015), which further interact with host factors (Figure 1.1). Furthermore, the diversity of the causes of BRD heightens the complexity of the disease, and this is reflected in its management and prevention.

Many studies have attempted to find a suitable way to effectively prevent BRD. Accordingly, vaccinations against bacteria and viruses have played a vital role through effectively enhancing resistance, albeit to a limited number of pathogens (Kasimanickam 2010). Chamorro and Palomares (2020) found that vaccination of calves with multivalent vaccinations alone or in combination with *M haemolytica* and *P multocida* at or shortly after weaning will effectively reduce BRD mortality morbidity after weaning. Moreover, antibiotics have shown an essential role in reducing the clinical signs of BRD during the infection. Nevertheless, the unregulated use of numerous antimicrobial drugs may lead to increased antibiotic resistance and ubiquity of respiratory pathogens. In addition, there is an increase in consumers' expectations for reduced antibiotic use (Urban-Chmiel and Grooms 2012). However, advances in managerial practices, vaccines, and clinical treatments, have done little to combat the extensive spread of pneumonia. There remains a critical need to enhance host resistance to pathogen colonization and pneumonia. Recently, more attention has been paid to early detection of BRD, starting with understanding the host innate immune response towards disease, and factors that could increase innate immune activity (Gershwin et al. 2015). Table 1.1 shows the characteristics of common viral pathogens associated with BRD.

Table 1.1: Characteristic Features of Major BRD Viral Pathogens.

Pathogen	Characteristics	Mechanisms of Pathogenesis	Effect on Host Response
Bovine Respiratory Syncytial Virus (BRSV)	Negative strand RNA virus, *Paramyxoviridae*	Entry through respiratory mucosa; infects bronchial epithelium, causes syncytial cell formation, bronchiolitis. Fever, cough, increased respiratory rate, depression	Immune modulation favoring T helper type 2 cytokines, which depresses cytotoxic T cell induction (Gershwin et al. 2000)
Bovine Herpes Virus 1 (BoHV-1)	DNA virus, Herpesviridae, *Alphavirinae*	Entry through respiratory mucosa; causes epithelial cell apoptosis. Fever, rhinotracheitis, cough, conjunctivitis, oral ulcers; reproductive tract infection with abortion.	Causes immunosuppression, and depresses interferon type 1 responses (Jones and Chowdhury 2010a).
Bovine Viral Diarrhoea Virus (BVDV)	Positive strand RNA virus, *Flaviviridae*, two biotypes 1 and 2	Spread in secretions; causes multiple system disease (abortion, persistent infection).	Causes immunosuppression, targeting and killing lymphoid tissue in Peyer's patches (Chase 2013)
Bovine Parainfluenza virus 3 (PI3)	Negative strand RNA virus, *Paramyxoviridae*	Entry through respiratory mucosa; infects bronchial epithelium, causes syncytial cell formation, bronchiolitis.	Causes immunosuppression, binds to sialic acid residues (Ellis 2010).
Bovine coronavirus (BoCV)	positive-sense, single-stranded RNA virus, *Coronaviridae*	Spread in secretions; causes calf enteritis and contributes to the enzootic pneumonia complex in calves. It can also cause winter dysentery in adult cattle.	Causes immunosuppression, (Ellis 2010).

1.2 Viruses as a causative agent of BRD

Occurrence of BRD may occur in animals of any age and at any stage of the production cycle (Gershwin et al. 2015). The disease most commonly affects young dairy and veal calves as well as cattle experiencing production cycle transitioning, such as at weaning or entrance into the farms(Klima et al. 2014).

Epidemiological aspects affecting BRD incidence include: microbial agents, transmission mode, microbial density, infectious and latent periods, and virulence of the relevant causative agents (Callan and Garry 2002). The most common viral pathogens concomitant with BRD are bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus3 (PI3), bovine adenovirus 3 (BAdSV), bovine viral diarrhoea virus (BVDV), bovine herpesvirus 1 (infectious bovine rhinotracheitis virus) (BoHV-1), and bovine coronavirus (BoCV) (Griffin 1997; Panciera & Confer, 2010; Pansri et al. 2020). Recent studies have utilized metagenomics to characterize further the virome of cattle afflicted with BRD. Ng et al. (2015) reported that in addition to previously documented viruses, bovine influenza D virus and bovine rhinitis A virus were also associated with BRD (Murray et al. 2016; Ng et al. 2015).

Both BRSV and PI3 are specific respiratory pathogens, while BoHV-1 and BVDV can affect various systems such as digestive and reproductive systems (Fulton 2009; Hay et al. 2016). These BRD-associated viruses replicate in the epithelial cells of the respiratory tract, causing mucosal inflammation, rhinitis, tracheitis, and bronchitis, allowing for adhesion and replication by pathogenic bacteria (Caswell 2014). Although BAdSV, BoCV, bovine influenza D virus, and bovine rhinitis A

virus were initially considered to be minor players in BRD, these viruses may have a pathogenic role rather than a strictly commensal one (Murray et al. 2016).

Viral pathogens can cause an initial infection that is frequently associated with mild clinical signs of BRD. The main role of the viral agents that are associated with BRD is causing immune suppression, which enhances susceptibility to secondary bacterial infections. BHV-1 and BVDV, BPI3V, and BRSV are spread via aerosolization, and all these viruses are considered to be major causes of BRD (Gershwin et al. 2015).

1.2.1 Bovine Respiratory Syncytial Virus (BRSV)

BRSV is a negative-sense single-stranded RNA icosahedral nucleocapsid virus that was first isolated in 1970 (Paccaud and Jacquier 1970). These viruses are relatively small (15kb) and belong to the *Paramyxoviridae* family, which cause similar lower respiratory tract diseases in a broad range of host species. The genome of the virus is translated into 11 proteins by 10 mRNAs. It has Polymerase protein (L), Phosphoprotein(P), and Nucleoprotein(N) proteins and the envelope of the virus contain three proteins which are Glycoprotein(G), Fusion protein(F) and Small hydrophobic protein (SH) that are associated with a matrix (M) protein (Figure **1.2**). Genomic RNA is a template for replication and transcription, with transcription occurring sequentially 3′ to 5′, and BRSV replicates in the cytoplasm of the host cell (Valarcher and Taylor 2007). BRSV is the most important respiratory pathogens in livestock worldwide (Valarcher and Taylor 2007). It can infect the calves and cause clinical signs in the absence of secondary bacterial infection (Larsen et al. 2001). Nevertheless, BRSV can cause immune suppression in infected

animals which can facilitate the proliferation of secondary bacterial infection in the lower respiratory tract leading to severe pneumonia. Therefore, the BRSV is the primary etiological agent of bovine respiratory disease complex, which causes high morbidity and mortality in cattle resulting in economic losses in cattle industry (Klima et al. 2014).

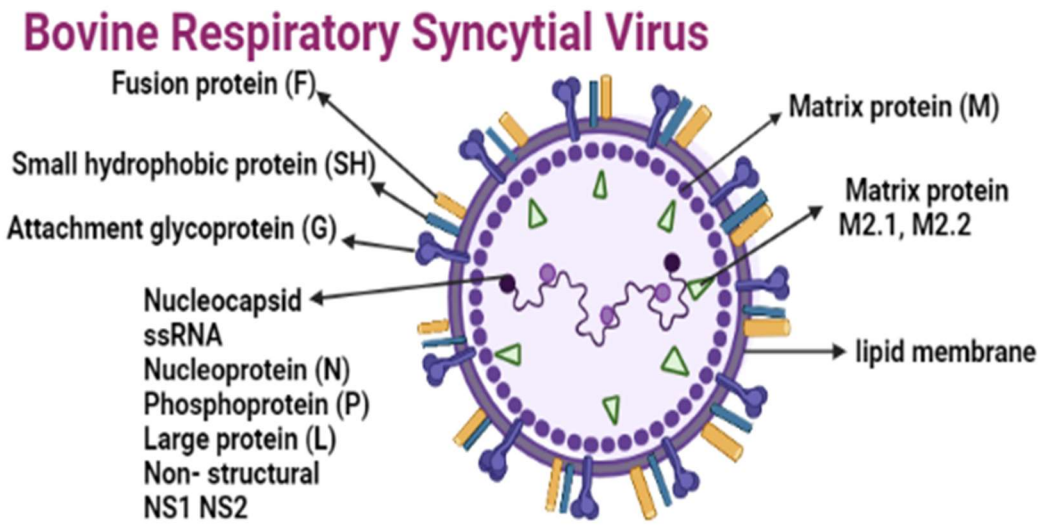

Figure 1.2:The structure of bovine respiratory syncytial virus. Modified figure from Valarcher and Taylor (2007).

1.2.2 Bovine herpesvirus 1 (BoHV-1)

The virus was first isolated in 1956 (Ellis 2009); BoHV-1, also known as infectious bovine rhinotracheitis virus (IBR), is a large (135kb), double-stranded DNA virus with an icosahedral nucleocapsid and envelope connected by matrix proteins. These viruses belong to the *Alphaherpesvirinae* sub-family, which, as a group, causes inflammatory disease in the upper respiratory tract in many host species. Temporally regulated gene sets encode more than 70 proteins, immediate-early (IE), early (E) and late (L), with some heterogeneity among members of the group.

The replication of these viruses takes place in the nucleus of the host cell (Ellis 2009).

BoHV-1 can affect various body systems such as the upper respiratory tract, genital tract, and conjunctiva, and may cause immunosuppression (Jones and Chowdhury 2010b), which can predispose to BRD. Primary cases of BoHV-1 result in mild clinical signs, and the animals will typically recover within 4 to 5 days unless the secondary bacterial infection contributes to the disease (Ellis 2009; Jones and Chowdhury 2010a). However, in many cases, BoHV-1 will leave the animals vulnerable to secondary bacterial infection and in adult animals a combination of abortion and respiratory disease can occur simultaneously (Jones and Chowdhury 2010b).

1.2.3 Bovine viral diarrhoea virus (BVDV)

BVDV, first isolated in 1946 (Olafson et al. 1946), is a positive-sense single-stranded RNA spherical virus in the genus *Pestivirus*. These viruses are relatively small (12.3kb) and belong to the family *Flaviviridae,* which as a group can infect various body systems (Brownlie et al. 1987). BVDV is divided into two genotypes, 1 and 2 (Ridpath et al.1994). Furthermore, within these two genotypes, the viruses are classified into cytopathic and non-cytopathic forms. In the case of BRD, BVDV genotype one and non- cytopathic forms have been isolated more than other forms in the US (Fulton et al. 2003).

BVDV is omnipresent in livestock and is a primary cause of bovine viral diarrhoea, which may infect various tissues, such as respiratory and reproductive tissues, leading to a range of clinical signs from mild and transient to fatal, with major economic losses such as abortion, infertility and reduced milk yield (Houe 2003).

These viruses have the ability to cross the placenta and infected the fetus with a noncytopathic form of BVDV in the first 125 days in utero, causing persistent infections (PI). Persistently infected cattle are the major factor in disease distribution in cattle populations worldwide (Brownlie et al. 1987) and, as such, PI can have a great effect on BRD incidence within a population (Shephard 2001). BVDV tends to be endemic in many cattle populations, approaching a maximum level of about 4% of the cattle being PI in the United States (Wittum et al. 2001).

1.2.4 Bovine Parainfluenza virus 3 (PI3)

PI3, first isolated in 1959 (McWilliam 1959), is a negative-sense single-stranded RNA virus with a spherical to pleomorphic shape (Kingsbury 1990). The virus is 150–200nm in size consisting of a nucleocapsid surrounded by a lipid envelope derived from the plasma membrane of the host cell from which it buds. The virus belongs to the subfamily *Paramyxovirinae,* order *Mononegavirales,* of the family *Paramyxoviridae,* which as a group, cause respiratory tract diseases in a broad range of host species. The genome of the virus is 15,456 nucleotides and comprises six structural proteins (N-P-M-F-HN-L) that encode for nine non-structural proteins (Ellis 2010) as shown in Figure 1.3.

Like other respiratory viruses, PI3 is adapted to allow infection of the respiratory system. On entering the respiratory tract, a PI3 virion would first encounter a mucous layer with a high content of N-acetylneuraminic (sialic) acid, a natural substrate for the neuraminidase activity of the HN glycoprotein in the viral envelope (Ellis 2010). Therefore, the virus causes degradation of mucus to allow penetration of the virus to target epithelial cells. PI3 also causes immunosuppression in infected

animals and facilitates the proliferation of the secondary bacterial infection in the respiratory tract leading to severe pneumonia (Ellis 2010).

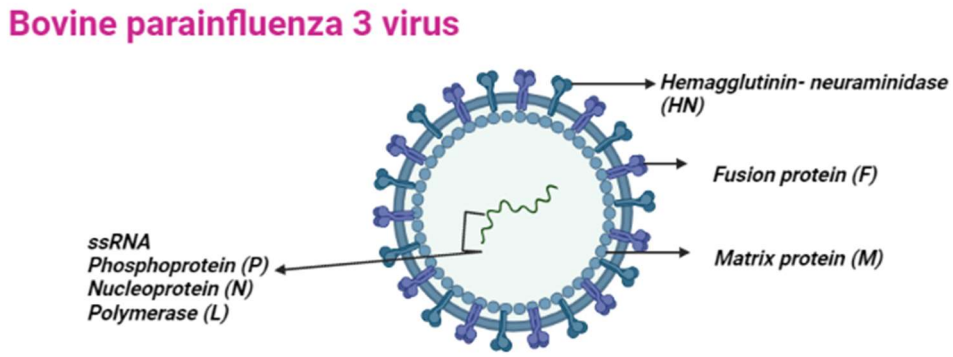

Figure 1.3 The major proteins of bovine parainfluenza virus 3. Modified figure from Philippe Le Mercier (2022).

1.3 Bacterial pathogens for BRD

Even though viral pathogens are still considered the primary cause of BRD, bacteria are also involved with BRD. Viral and bacterial pathogen combinations compromise the respiratory tract defences, ultimately leading to pulmonary disease. BRD is caused by environmental and host factors which contribute to allow infection by common pathogens (Grissett et al., 2015).

Bacterial pathogens are of particular interest in the farming of dairy and beef calves. Common bacterial pathogens associated with BRD are: *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis*, *Histophilus somni*, and *Trueperella pyogenes* (El-Deeb et al., 2020; Gershwin et al., 2015; Murray et al., 2017; Pansri

et al., 2020; Urban-Chmiel & Grooms, 2012). These bacterial pathogens (summarised in Table 1.2) are omnipresent in the cattle herds and present as normal commensals in the upper respiratory tract of cattle. Bacteria can proliferate and be inhaled into the lungs and cause substantial morbidity and mortality under appropriate environmental conditions such as stress, excessive heat and cold, poor ventilation and post-viral infection (Urban-Chmiel & Grooms, 2012; Grissett et al., 2015). Each bacteria has different virulence factors, including adhesins, biofilms, capsules, enzymes, and toxins, that increase its ability to colonies the lower airway, avoid the immune system, resist antimicrobial treatment, cause tissue damage, and induce an intense inflammatory response (Panciera and Confer 2010).

Table 1.2: Characteristic Features of Major BRD Bacterial Pathogens.

Pathogen	Characteristics	Mechanisms of Pathogenesis	Effect on Host Response
Pasteurella multocida	Gram negative coccobacillus, family *Pasteurellaceae*	Part of normal microbiota in upper respiratory tract; stress or viral infections allow it to infect lung and cause bronchopneumonia.	Multiple virulence factors: anti-phagocytic capsule, protein toxin (Dagleish et al. 2010).
Mannheimnia hemolytica	Gram negative coccobacillus, family *Pasteurellaceae*	Upper respiratory tract commensal; opportunistic pathogen causing bronchopneumonia.	Multiple virulence factors: adhesin, capsular poly-saccharide, leukotoxin, transferrin binding protein (Singh et al. 2011)
Mycoplasma bovis	Wall-less bacterium of class Mollicutes	Causes mastitis, arthritis, otitis media, pneumonia, fever, cough, anorexia, nasal discharge; synergistic with other BRD pathogens, forms biofilms to facilitate persistence	Variable surface membrane, adhesins, inhibits neutrophil respiratory burst (Caswell et al. 2010).
Histophilus somni	Gram negative coccobacillus, *Pasteurellaceae*	Upper respiratory tract, reproductive tract commensal; Diseases: thrombotic meningoencephalitis (TME), respiratory disease, myocarditis, polysynovitis, otitis media, mastitis, and reproductive tract diseases.	virulence factors including surface proteins, binding to and induction of apoptosis in host endothelial cells that allow the bacteria to colonize host tissues (Corbeil 1996).
Trueperella pyogenes	Gram-positive, appear as pleomorphic or coccoid rods	Mucous membranes of the upper respiratory, gastrointestinal, or urogenital tracts of animals	Virulence factors, extracellular matrix-binding proteins, fimbriae contribute to the adhesion and colonization of the host tissues (Swida and Stefa 2019)

1.4 Pathogenesis of BRD

The pathogenesis of BRD frequently includes complex interactions between environmental factors, pathogens, and the animal's immune response (Murray et al. 2016). After the transmission by direct contact with nasal secretions or aerosols of infected animals over a short distance, pathogens, especially viruses, spread to various ciliated and non-ciliated epithelial cells in the respiratory tract, including the airways and pulmonary parenchyma (Ellis 2009; Grissett et al. 2015). Viruses play a significant role in creating an environment favourable to the colonisation and replication of pathogenic bacteria, leading to pneumonia. Viruses can cause mucosal surface alterations such that bacterial adhesion to virus-infected cells is enhanced; further colonisation occurs more readily than in intact mucosa in areas of virus-induced mucosal erosion (Grissett et al., 2015). Moreover, viral pathogens could also play an essential role in the immune system, manipulating innate and adaptive mechanisms through changes in the function of alveolar macrophages, suppression of proliferation of lymphocytes, induced apoptosis, and cytokines and other inflammatory-mediated releases (Jones and Chowdhury 2010a). This combination of factors produces respiratory disease of variable clinical signs in uncomplicated infections and can predispose the lungs to secondary bacterial infections typical of BRD if viral replication is not controlled (Patel et al. 2017; Urban-Chmiel & Grooms).

1.5 Clinical and subclinical signs of BRD

Clinical and subclinical BRD is a result of immunosuppressive stressors allowing respiratory tract colonisation by opportunistic pathogens commonly encountered as normal commensals in the upper respiratory tract (Klima et al. 2014). These

physical, environmental, and epidemiological stressors are exacerbated during weaning and farm production stages, leading to increased incidence of clinical BRD, as discussed above (Rice et al. 2007). Acceptance on an industry-wide basis that a high percentage of animals will become infected with BRD makes accurate and proper diagnosis essential (Poulsen and McGuirk 2009). Most cases are identified and treated prior to the first 27 days of the feeding period (Buhman et al. 2000; Grissett et al. 2015; Wolfger et al. 2015).

Depression is the most common and earliest recognisable clinical sign of pneumonia (Buhman et al 2000). Calves with depression will have drooping ears, an extended head, a bent back, and are frequently separated from other cattle (Statham 2013). Following that, infected calves become sicker, their respiratory rate increases, and they will stop feeding and become dull (Duff and Galyean 2007). These clinical signs are due to their high temperature, which often reaches 40 – 42°C. Moreover, Lungs sounds increase and can be heard with a stethoscope; if left untreated, the calve typically die within 24 to 48 hours (Urban-Chmiel and Grooms 2012; Grissett et al., 2015; Duff and Galyean 2007).

The chronic form of BRD is usually recognised by a severe cough if the calf pneumonia is not treated (Duff and Galyean 2007). Presentation of clinical signs may vary between individuals, making the diagnosis of BRD difficult. Duff and Galyean (2007) recommend that observation of any combination of the clinical signs described above, including a rectal temperature of over 40°C , is indicative of BRD. Given the subjective nature of the diagnosis, accurate identification of infected animals is not always possible. The animal's clinical signs may present subclinically and therefore go undetected by farm personnel (Duff and Galyean 2007). It is not well known why, when infected, some individuals within

populations present clinical signs and others present subclinically. Noffsinger and Locatelli (2004) attributed subclinical expression of clinical signs to a predator/prey response. They proposed that animals would suppress their clinical signs so as not to be perceived as weak in the presence of a potential predator.

1.6 Laboratory diagnostic tests for BRD

Traditionally, farm staff usually assess cattle's health based on their appearance and behaviour with clinical scoring system shown in Figure 1.4. According to their protocol, animals with total clinical score of ≥5 should receive therapeutic treatment (Perino and Apley 1998). However, this method has insufficient sensitivity, with an estimated 62% detection rate in identifying BRD (White and Renter 2009). One of the limitations of clinical scoring system diagnosis is the natural behaviour of cattle, which is expressed in response to human presence, especially at a young age. Cattle may hide clinical signs of the disease from a human, which results in less sensitivity of diagnosis (White and Renter 2009). Moreover, recently there are many studies have used thoracic ultrasound scoring as an accurate tool for detecting BRD in dairy calves (Rhodes et al. 2021). Therefore, BRD in cattle may either be detected late or not identified at all. BRD usually leads to high morbidity and mortality rates, hence, early intervention is necessary for effective treatment (Timsit et al. 2011).

The fluorescent antibody test (FA) is a direct lab analysis test that uses a fluorescent dye to determine the infective agents (Devi and Mui 2010). Although FA testing is used in diagnosing most BRD viruses in tissues, the sample obtained in the diagnosis process cannot be kept for an extended period due to the fast decay of the dye activity used in the diagnosis.

The immunohistochemistry (IHC) test is recognised as an appropriate diagnostic tool to be used in veterinary laboratories. According to Narita et al. (2000) IHC can be used in the identification of bovine herpes virus present in cells acquired by bovine bronchoalveolar lavage. IHC is mostly used in skin biopsies to detect BVDV antigen (Grooms and Keilen 2002). It is a reliable test, and calves recently vaccinated with modified live vaccines did not cause false positives (DuBois et al. 2000).

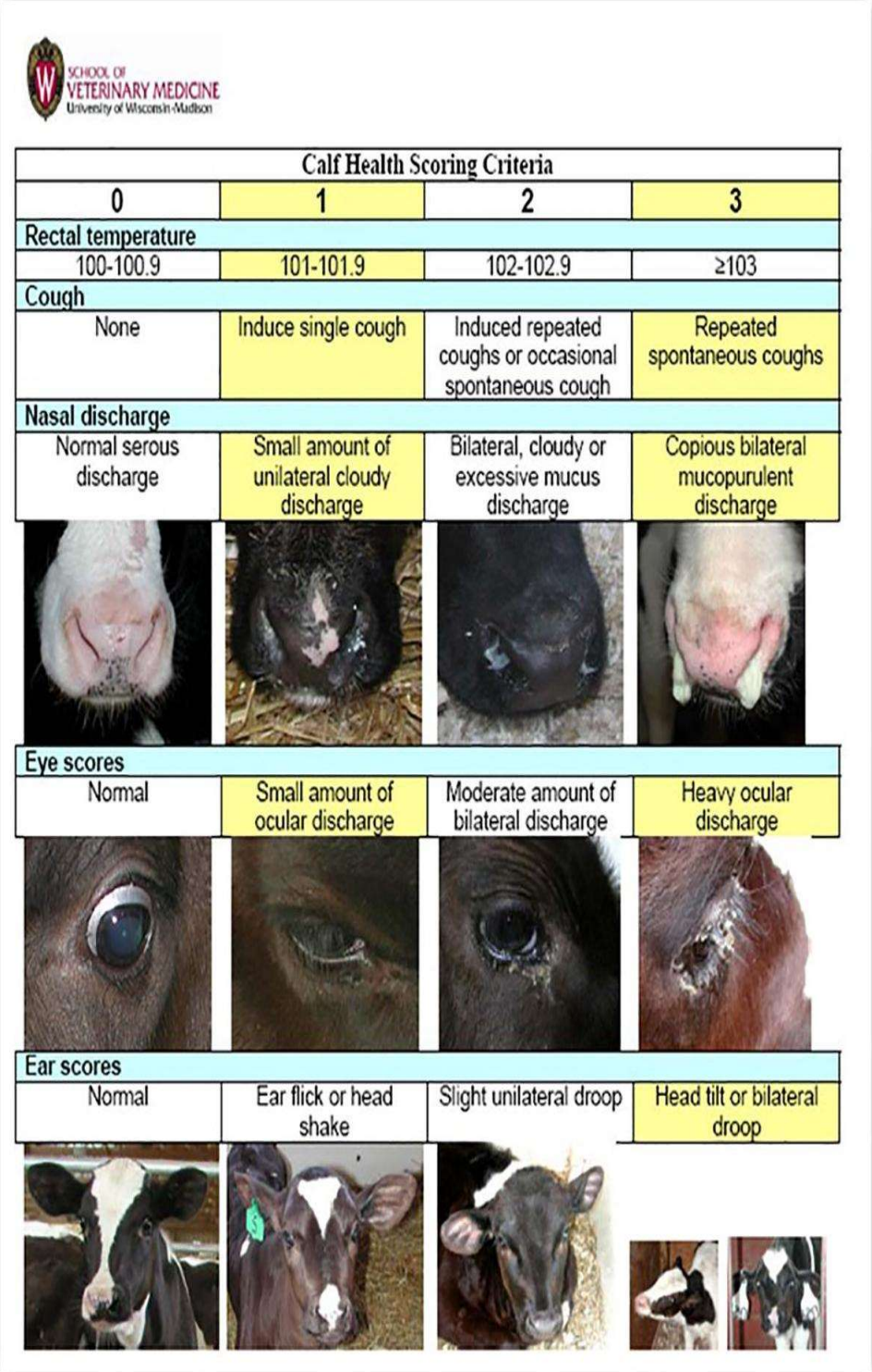

Figure 1.4 : Clinical scoring system used to assess general health of cattle. The figure was designed by researchers at the University of Wisconsin at Madison. Unmodified figure (University of Wisconsin-Madison). Total scoring 4 watch, and ≥5 indicated an urgent need for treatment.

On the other hand, multiple BRD predictive tests can be used to provide valuable information when linked with a thorough physical examination. In past decades, the white blood cell (WBC) count, in particular neutropenia, left shift neutrophilia, lymphopenia, or increased neutrophil/lymphocyte ratio, was used to identify severe or mild inflammation in cattle (Jones and Allison 2007). Also, positive acute-phase proteins such as serum amyloid A, haptoglobin (HAP), apolipoprotein AI, fibrinogen, and lipopolysaccharide-binding protein (LB) increase in cattle that have inflammation and tissue damage and a decrease in negative acute-phase proteins such as transferrin. Variation in acute-phase proteins is connected with BRD (Aich et al. 2009; Jones and Allison 2007). In addition, stress-related proteins, such as cytokines, stimulate the hypothalamic-pituitary-adrenal axis and increase the peripheral glucocorticoid concentration (Schaefer et al. 2012). Blood cortisol concentrations measured in cattle are used to diagnose BRD (Schaefer et al. 2012). The pathogen and biomarkers are detected neopterin procalcitonin, serum amyloid A, haptoglobin, proinflammatory cytokines – and infected calves have significantly greater serum values of these compounds compared to healthy calves (El-Deeb et al. 2020). Microbial culture can be used to isolate and identify the bacteria from nasal, nasopharyngeal swabs, transtracheal wash, or lungs necroscopy (Fulton & Confer, 2012). Moreover, Pen-side test would facilitate more rapid diagnosis test for many pathogens associated with BRD (Sachse et al. 2018). However, none of these tests are specific to inflammation associated with BRD.

1.6.1 Molecular diagnosis of BRD

The primary application of molecular technology in microbiology is identifying pathogen nucleic acid DNA or RNA, bacterial genera, species and subspecies, and

viral genotyping (Veir and Lappin 2010). These tools permit laboratories to recognise viruses and bacteria rapidly without requiring additional time-consuming cell culture, incubation or biochemical tests. Several laboratory methods for BRD detection are available. These methods include identifying the causative viral or bacterial pathogen using PCR and real-time PCR assays (Duff and Galyean 2007; Urban-Chmiel and Grooms 2012).

Polymerase chain reaction (PCR) for the causal diagnosis of BRD is now very popular. Additionally, there are specific PCR assays for all the pathogens that are involved in BRD (Amer et al. 2013, Rahpaya et al. 2018). PCR amplifies a small, short, and distinct portion of a DNA strand that can either be a particular gene or part of a gene (Rahman et al., 2013). In contrast to living organisms, the PCR assay copies only tiny DNA fragments and uses up to 10 kilobase (kb) pairs, whereas specific techniques can copy fragments up to 40kb. The PCR reaction is conducted in a thermal cycler, a machine that heats and cools the reaction tubes to the specific temperature required for each reaction step (Rahman et al., 2013).

The DNA fragment of interest is amplified by including specific primers in the reaction mixture. Primers are artificial nucleotide strands, generally short with less than fifty nucleotides. Any given PCR will contain forward and reverse primers, that respectively, complement the start and end of the gene to be amplified (Rahman et al., 2013). Hence, the primers adhere to the start and end of the DNA template, and the DNA polymerase binds and begins the synthesis of the new DNA strand. In general, the PCR process comprises approximately 20 to 35 cycles, and each cycle involves three steps (Rahman et al., 2013):

1. The denaturing process to separate the double-stranded DNA is usually heated at an average temperature of 94–96°C. The process involves the breakdown of the hydrogen bonds that join the DNA strands together. Before starting the first phase, the DNA must be fully denatured to ensure that both the primers and the template DNA have entirely separated and are only single-stranded. The denaturing process requires about two to five minutes, and the DNA polymerase depends on the polymerase being used and will be activated in the process (Rahman et al., 2013).

2. The denaturing process is followed by the annealing process. The temperature is lowered after separation of the DNA strands to enable the primers to attach themselves to the single strands. The temperature in this stage must be below the primers' melting point and thus ranges from 45°C to 60°C. A higher or lower temperature at this stage prevents the primers from binding to the template DNA or makes them bind randomly, respectively. The annealing steps may require up to one minute (Rahman et al., 2013).

3. The extension step begins at the annealed primers and works its way along the DNA strand, allowing the DNA polymerase to fill in the missing strands. The temperature in this cycle is dependent on the DNA polymerase, though is typically around 72°C. Time also depends both on the length of the amplified DNA fragment, and the DNA polymerase, and as a rule-of-thumb, represents one minute per kbp (Rahman et al., 2013).

RT-PCR (reverse transcription PCR) may be used to isolate, amplify, or identify a known sequence from a cell or tissue RNA. The RT-PCR is similar to the standard PCR but includes an initial step where RNA is copied to complimentary DNA (cDNA) by the enzyme reverse transcriptase. This method is extensively applied

in expression mapping and determining where and when specific genes are expressed (Rahman et al., 2013) and is commonly used to detect viruses with an RNA genome.

Quantitative PCR (qPCR) uses fluorescent dye labelled probes to measure the amplified product in real time. qPCR is also known as quantitative real-time PCR or real-time PCR. It is often the most appropriate technique for measuring the amount of DNA or RNA (when preceded by reverse transcription) present in a sample. Therefore, qPCR is an indirect method used to quantitatively measure the initial amounts of DNA, RNA, or cDNA. Quantitative PCR is among the essential techniques used in medical and veterinary diagnosis and for research. This molecular technique provides a rapid, specific, and sensitive method for detecting and quantitating pathogens (Holland et al. 1991; Livak et al. 1995). It is commonly used to determine whether a sequence is present, and the number of copies presents in the sample. (Rahman et al., 2013).

Quantitative PCR (qPCR) can detect and quantify the viral and bacterial species' density in the samples. Thomas et al. (2019) used a qPCR assay to target the presence of BRD associated bacteria in 299 cow nasal swabs and quantify their carriage over time. In nasal swabs, Pasteurella *multocida* was detected (75%), *Histophillu somni* (26.8%), and *Mannhemia haemolytica* were found (5.7%). In addition, the carriage density of *H. somni* in the majority of the swabs was between 10-100 genome copies/ml (82.5%). The carriage density of *M. haemolytica* was between 100 and 1,000,000 genome copies/ml, while *P. multocida* was between 1,000 and 100,000 genome copies/ml. Also, a study conducted using qPCR to detect 3 types of viruses BoHV-1, PI3 and BRSV in 541 calves using swabs and

bronchial alveolar lavage samples. They have found that 8% of samples were positive for BoHV-1, 2.5% and, 5% of samples were positive for PI3 and BRSV, respectively (Thonur et al. 2012).

Nevertheless, laboratory analysis of multiple BRD pathogens in different assays is very costly for livestock producers, so recently, a multiplex PCR assay has been developed (Pansri et al., 2020; Horwood and Mahony, 2011). Although individual bacterial or viral pathogens can cause disease, mixed infections are the most common and problematic in BRD. A rapid diagnosis of these pathogens would enable the rapid implementation of specific treatment and control measures. A multiplex qPCR method to detect relevant viral pathogens of BRD was recently developed, demonstrating better performance than culture in co-infections with BoHV-1, BVDV, and PI3 (Horwood and Mahony, 2011; Pansri et al., 2020). In another study, multiplex quantitative PCR (qPCR) was used specifically to detect and quantify the viral (PI3, BCoV, BRSV, BoHV-1, and BVDV) pathogens most frequently associated with BRD (Pansri et al., 2020). A multiplex quantitative PCR (qPCR) is fast diagnosis methods during BRD outbreak.

1.7 Preventative and management strategies for BRD

Because BRD manifestation results from complex interactions between environmental, pathogen, and host-related factors, preventative measures and best management practices also require a multifaceted approach to reduce BRD prevalence (Schaefer et al. 2012). Vaccination protocols, nutritional status, cattle temperament, ventilation, castration, dehorning, and general cattle handling

practices should all be considered when developing BRD prevention strategies (Urban-Chmiel and Grooms, 2012).

Most of the research has been directed towards pharmaceuticals and management practices, as these are currently the easiest methods to implement prevention. The selection of antibiotic treatment is based on veterinary advice and experience of previous respiratory disease outbreaks in the area (Urban-Chmiel and Grooms, 2012; Booker and Lubbers 2020). Furthermore, it is usual for bacterial infections to reappear again in the same herds (up to 25% of bacterial infection recurrences of a previous infection), often requiring repeated antibiotic treatment two weeks later (Booker and Lubbers 2020). Therefore, it is vital to note that a failure of antibiotic therapy does not cause this condition, rather re-infection of the physically weakened respiratory tract occurs several days after antibiotic levels have dropped below effective levels (Booker and Lubbers 2020). However, there is increasing pressure to reduce the use of antibiotics in farming due to the risk of antimicrobial resistance (NADIS, 2020).

1.8 Predisposing factors for BRD

Infection with BRD can occur in cattle at any stage of life (Murray et al. 2017). Dairy and beef calves are more susceptible to disease, especially under intensive production systems or associated with stressors such as weaning, castration, shipping, and crowding (Duff and Galyean 2007). BRD is a multifactorial disease, involving multiple predisposing factors that work synergistically with bacteria and viruses, contributing to develop BRD clinical signs (Duff and Galyean 2007; Guterbock 2014). Poor ventilation in calf housing is the critical environmental risk factor predisposing calves to BRD (Pritchard 1982; Patel et al. 2017). Furthermore,

outbreaks of pneumonia in young calves have also been associated with cold, humid conditions, sudden changes in air temperature, stress due to multiple factors, and environmental changes (Phillippo et al. 1987; Gorden and Plummer, 2010).

Host factors could include gender, age, breed, animal immune status, genetics, and concurrent illness (Grissett et al., 2015). The risk of BRD is often greater under increased stress due to poor management practices. These management practices include weaning conditions, transport, diet change, high stocking density, handling, and surgical procedures (dehorning, castration). In addition, mixing of livestock leading to changes in the social system is detrimental (Duff and Galyean 2007). Although a substantial body of literature supports the association between BRD and these predisposing factors, there are many challenges to effective field research surrounding BRD that make it difficult to establish causal relationships. Increased stress resulting from transportation is one of the leading contributors to the incidence of BRD. Transportation is the greatest identified non-infectious risk factor leading to BRD and the reason that BRD is often referred to as "shipping fever" (Urban-Chmiel & Grooms). Various aspects of transportation (e.g., loading and unloading, the duration and method of transport) have been studied to identify which transportation component has the greatest impact (Dixit et al. 2001; Martin et al. 1988). Table 1.3 illustrates some of the multifaceted effects of BRD incidence in both the preweaning and postweaning phases of production.

Table 1.3: pre-and post- weaning factors affecting bovine respiratory disease complex (BRD) in beef cattle and the resulting outcomes of the disease. Modified from Duff and Galyean (2007).

Preweaning factors	Postweaning factors
Prenatal nutrition	Transportation and marketing stress
Intake of colostrum	Commingling
Persistent of Bovine viral diarrhoea	Prophylactic antibiotics
Preweaning health	Receiving diet nutrients
Temperament	Receiving period managmet
Preshipment management -Preconditioning - Vaccinations - Nutritional status	-Castration -Dehorning -Implant programs

1.8.1 Environmental factors

Environmental risk factors have also been associated with risk of BRD. Building design, ventilation, air quality, humidity, temperature inside the farm unit and seasons have been linked to BRD incidence (Hay et al. 2016). The seasons are one of the most crucial factors that contribute to the incidence of BRD. BRD shows increased incidence in autumn and summer which was reported by North American studies (Hay et al. 2016). Another study by AlHammadi (2016) in Saudi Arabia shows that BRD is increased in north Saudi Arabia because it is relatively cold. One possible explanation for this is that when the weather changes suddenly, whether cold or hot, will reflect on the calves' healthy and leave them susceptible to BRD. Furthermore, proper ventilation decreases pathogen concentration and noxious

gases as well as refreshing the air in the animal unit (Sowiak et al, 2012). Therefore, the ventilation systems must always provide a continuous flow of fresh air to every housed animal. There are different systems to provide proper ventilation: natural and mechanical ventilation, and all these systems should consider different seasons to avoid overheating or cold (Sowiak et al, 2012). There are differing recommendations for minimum ventilation rates of calf barns. For mechanically and naturally ventilated is recommended ventilated 4 air changes per hour (Nordlund and Halbach 2019; Midwest Plan Service, 1990). Many studies have focused on natural ventilation of cattle farms in order to reduce the cost of both electrical power bills and fans (Lago et al. 2006; Nordlund and Halbach 2019).

Regarding the temperature, which is a very important factor in calves' health, very few researchers have studied the effects of heat stress on the calves. The optimum temperature depends on many factors such as; air speed, floor type, humidity and outer temperature (Nordlund and Halbach 2019). There is no specific recommendation and guideline for the barn temperature, whether in cold or hot weather, except 1 study by (Nordlund and Halbach 2019), that recommended temperature on the farm unit is between 4.5°C and 15°C for calves with floor bedded with straw.

1.9 Management practices to prevent BRD

Management practices that minimise the entry, exposure, and transmission of pathogens are essential steps in BRD management (Snowder et al. 2006; Grissett et al., 2015). Additional management practices associated with the development of

BRD include ventilation, nutritional management, and cattle handling. Prior to a BRD challenge, the herd's nutritional status plays a vital role in the proportion and outcome of animals infected (NADIS, 2020).

Cattle handling and cattle temperament are important considerations when discussing BRD management, as both impact stress levels of cattle. There is strong evidence to support the negative effect of stress on cattle health and performance. Utilising low-stress cattle handling practices can reduce the risk for BRD (Hodgson et al. 2005). Fell et al. (1999) evaluated differences in BRD treatments in cattle that were designated as 'calm' or 'nervous'. They reported that cattle in the nervous group required a greater number of treatments for BRD compared to the calm group.

Ventilation plays a vital role in animal management, and poor ventilation is one of the most significant factors affecting respiratory health (Nordlund 2008). Proper ventilation leads to decreasing concentrations of airborne pathogens, noxious gases, airborne dust contamination, and airborne endotoxin levels; helps to maintain an optimum ambient temperature and humidity levels; and eliminates areas of stagnant air (Callan and Garry 2002; Quintana et al. 2020). The reduction in the concentration of airborne pathogens is one of the most significant aspects of proper ventilation (Quintana et al. 2020). Pathogens, including viruses and bacteria, can reach a high concentration in poorly ventilated farms and spread quickly (Callan and Garry 2002). Therefore, enhancing ventilation is one of the crucial elements to minimise the concentration of pathogens in farm animals.

Calves reared in outdoor systems are at a lower risk of pneumonia before the weaning period than those born and reared indoors (Lorenz et al. 2011). Furthermore, passive immunity is essential for calves, which is acquired from the

colostrum or maternal antibodies crossing the placenta to the fetus *in utero*. Colostrum is rich in antibodies, also known as immunoglobulins, and the calf needs to have adequate amounts before its immune system is fully functional. It is highly recommended to feed the calf in the first 24 hours of birth from three to four litres of colostrum to support the immune system (Besser et al. 1991). As the highest occurrence of BRD occurs during the first three months in many herds, prevention should focus at this time on reducing stress. Close contact with other animals enables the fast dissemination of respiratory pathogens; individual housing of dairy calves is commonly associated with better calf welfare, either indoors or outdoors (Lorenz et al. 2011).

Although environmental and management stressors play a major role in BRD incidence, there is an increasing body of evidence to support that BRD susceptibility is partially subject to genetic influence. The selection of less susceptible animals to BRD offers a viable method for reducing BRD prevalence (Snowder et al. 2006). Heritability estimates for BRD susceptibility range from low (0.04%) to moderately (0.26%) heritable (Muggli-Cockett et al. 1992, Schneider et al. 2009). Furthermore, Snowder et al. (2006) reported that BRD incidence was heritable by genetic variation within and between breeds. Nevertheless, it should be recognised that chronic pneumonia in calves is a problem for many herds. It is essential to try to determine the causes of the disease, whether genetic or bacterial and viruses, when the condition becomes severe enough to warrant medication (Urban-Chmiel and Grooms 2012). Improvement of the management and environment or eradication with a comprehensive disease control plan of the causative agents is essential.

1.9.1 Vaccination for BRD

Vaccination prevents infections and likely deaths in millions of animals globally. Vaccines contain pathogens (virus or bacteria) that are typically of reduced virulence, or parts of pathogens such as protein or toxin, that is injected into the animal's body to promote an immune response and prevent subsequent disease from occurring (Boshra et al. 2016). Furthermore, vaccinations can be attenuated vaccines, which contain a viable organism of reduced virulence, or inactivated vaccines, which contain only an antigen of a killed pathogen. Moreover, a vaccine can be monovalent or multivalent; a monovalent vaccine contains one pathogen, whereas a multivalent vaccine may contain three or four pathogens (Paton and Taylor 2011).

Vaccination against both viral and bacterial pathogens can reduce the risk of developing BRD. Vaccines can be effective for reducing both susceptibility and the shedding of infectious pathogens to other calves (Frank et al. 2002), and vaccination against the various pathogens involved in BRD is an important part of any prevention program. In the United Kingdom, vaccines against the viral pathogens BoHV-1, BVDV, PI3, and BRSV and the bacterial pathogens *M. haemolytica*, *P. multocida*, and *H. somni* are readily available. Most of these vaccines are multivalent; that is, they contain multiple agents, intending to reduce the number of injections, broaden protection, and reduce the overall cost (Sherwin et al., 2018). Viral vaccination will prevent virus infection, reducing the risk of immunosuppression, which means that the bacteria will not have a suitable environment in which to flourish. As viral pathogens are the leading cause of BRD, vaccination is essential in prevention because antibiotics are not effective against viruses (Urban-Chmiel & Grooms, 2012). However, if BRD develops suddenly,

antibiotics are crucial in minimising infection and clinical signs (Duff and Galyean 2007). One limitation of using antibiotics as a preventive measure against BRD is that bacteria can become resistant and do not respond to antibiotics if the animal is exposed to other infections (Duff & Galyean, 2007). A summary of the currently available vaccines is given in Table 1.4.

Table 1.4: Multivalent bovine respiratory disease complex vaccines available in the UK. Modified from Sherwin et al. (2018).

Vaccine	Producers	Disease	Volumes of vaccine, route of administration and active substances	Volume's schedule and booster	Duration of immunity
BOVALTO Respi 4	Boehringer Ingelheim	parainfluenza type 3 Bovine viral diarrhoea Bovine respiratory syncytial virus *Mannheimia haemolytica* serotype A1	2ml, Subcutaneous, Inactivated vaccine	From two weeks of age: two injections three weeks apart, booster after 6 months.	six months
Rispoval 4	Zoetis	parainfluenza type 3 Bovine viral diarrhoea Bovine respiratory syncytial virus Infectious bovine rhinotracheitis	5ml, Intramuscular, (live attenuated vaccine parainfluenza type 3 and Bovine respiratory syncytial virus), and (inactivated Bovine viral diarrhoea Bovine and Infectious bovine Rhinotracheitis)	From three weeks of age, vaccinate at three, six, and 12weeks, booster after 6 months.	six months
Hiprabovis Somni/Lkt	Hipra	*Mannheimia haemolytica* serotype A1 *Histophilus somni*	2ml, Subcutaneous, Inactivated vaccine	From two months of age and give a second dose after 21 days, booster not stated.	Not provided
Rispoval intranasal RS + PI3	Zoetis	parainfluenza type 3 Bovine respiratory syncytial virus	2 ml, Intranasal, live attenuated vaccine	From nine days of age with a single dose, booster after 3 months.	12 weeks

Bovilis Bovipast RSP	Intervet	parainfluenza type 3 Bovine respiratory syncytial virus *Mannheimia haemolytica* serotype A1	5ml, Subcutaneous, Inactivated vaccine	From two weeks old: two doses at a four-week interval, Boosters to be given two weeks before stressful event.	Not provided
BOVALTO Respi 3	Boehringer Ingelheim	parainfluenza type 3 Bovine respiratory syncytial virus *Mannheimia haemolytica* serotype A1	2ml, Subcutaneous, Inactivated vaccine	Calves can be vaccinated from 2 weeks of age. Calves from non-immune dams: 2 injections 3 weeks apart from 2 weeks of age	six months
Bovalto Respi Intranasal 5	Boehringer Ingelheim	parainfluenza type 3 Bovine respiratory syncytial virus	2ml intranasally (1ml of the vaccine per nostril) to calves from 10days of age using an intranasal applicator. live attenuated vaccine	Calves from age of 10days	12 weeks after vaccination
Hiprabovis SOMNI	HIPRA	*Mannheimia haemolytica* serotype A1 and *Histophilus somni*	2ml, Subcutaneous, Inactivated vaccine	Calves from 2 months of age dose should be repeated after 21 days	six months

1.10 Methods for sampling the environment to detect viral and bacterial pathogens

1.10.1 Air sampling for viral or bacterial culture

The MD8 air sampler is a modern portable air sampling device commonly used for collecting airborne pathogens, including viruses. It has been widely used, and the results show that it offers 100% sensitivity for RNA viruses (Zhao et al. 2014b).

The device works using filtration techniques, which allow air to pass through the gelatine filter. The gelatine filter is 80mm in diameter and comes with a specific pore size of 3µm which captures both viruses and bacteria. Additionally, the gelatine filter is soluble in viral transport media and in agar for the bacteria (Kim et al. 2016).

There are specific, and non-specific cultures and media used to transport bacteria and viruses and keep them viable for laboratory analysis. The viruses media contains an isotonic solution and protective protein, antibiotics to control microbial contamination, and one or more buffers to maintain the pH (Verreault et al. 2008; Kim et al. 2016). This virus media can be used to dissolve samples collected onto a gelatine filter, which is water-soluble. Additionally, the bacteria can be grown in artificial media such as nutrient broths and agar plates (Johnson 1990). For an example of transport media, a study by Verreault et al. (2008) used a PTFE filter, which dissolved in 30 ml of viral transport medium. This medium contained a sterile phosphate buffer with 10% foetal calf serum, 10,000U/mL penicillin, 10mg streptomycin, and 25µg amphotericin B. All samples should be stored in the refrigerator immediately at 4°C if not analysed directly. If being stored for a long time, they should be kept at -80°C until viral or molecular biological analysis (Kim et al. 2016), using techniques such as quantitative PCR (discussed above).

1.10.2 Sampling time and flow rate

Airborne pathogens, including viruses, are usually present indoors more than outdoors (Popescu et al., 2011). Outdoor bioaerosol sampling is conducted in occupational environments such as animals' farms and polluted environments. Furthermore, airflow rates are important to calculate the concentration of pathogens

in the air, and usually, the flow rate comes with the manufacturer's specifications (Jensen et al. 1998). An example of an outdoor air study was conducted by Dong and Yao (2010), who used an air bio sampler and gelatine filter with an average flow rate of 12.5L/min for 30min to detect bacteria and fungus in indoor and outdoor areas, and it was found that there were more present in the outdoor area. Moreover, an indoor air study by Kim et al. (2016) used an MD-8 air scan sampling machine for detecting the virus with a flow rate of 50L/min for 20min, and the results show that four of seven air samples were PCR positive for the MERS coronavirus in indoor air of hospitals. In another study on virus detection, Liu et al. (2020) used a gelatine filter with a flow rate of 5L/min for 1h and found that the virus was concentrated in some areas, such as toilets. It seems that the MD-8 air scan sampling machine is commonly used with a gelatine filter and showed an appropriate performance for sampling airborne viruses.

1.11 Conclusion

Despite widespread use of antibiotics and vaccination to prevent the BRD, the disease continues to threaten the global cattle industry. The majority of studies on vaccinations and antibiotics of infected and susceptible animals with BRD were conducted 10 to 20 years ago. Presently, relying on old literature may undermine our understanding of the prevention of BRD. There is thus a limitation of recent research on the optimal strategies for disease prevention. Furthermore, the required conditions for minimising disease spread (e.g., temperature, humidity) are not clear. The relative importance of direct, aerosol or fomite transmission of infectious agents is poorly understood. Therefore, this study aims to examine, detect, and

quantify respiratory viruses and bacteria in airborne samples and relate the data to environmental conditions, including temperature, relative humidity and calf number.

1.12 Aim and Objectives

The aim of study was to detect and quantify airborne viral and bacterial pathogens associated with bovine respiratory disease complex.

(i) Determine the optimum conditions for the use of two different air sampling machines, one that collected air directly on to plates for bacterial culture, and the other that collected air onto gelatine filters. [pilot study]

(ii) Quantify airborne viruses (BoHV-1, PI3 and BRSV) and Gram negative and Gram-positive bacteria in air samples. [longitudinal study]

(iii) Investigate whether environmental conditions, including temperature and relative humidity, might be risk factors for presence of pathogens involved in BRD complex. [longitudinal study]

Chapter 2: General Materials and Methods

2.1 Introduction

The general materials and methods used in the study are described in this chapter. Following that, specific methods are discussed in the appropriate chapters.

2.2 Barn selection

We used a number of criteria to select barns for this study, including natural ventilation (with or without a positive ventilation tube), history of calf pneumonia and a minimum of 15 pre-weaned calves in the same barn. These criteria were chosen such that we would expect BRD cases to occur during the study. Therefore, two farms were chosen because they only achieved the criteria of this study with consideration that there are no significant changes in the barn, such as moving the calves or changing the ventilation system, before and during sample collection. Therefore, this study used two farms, one at the Centre for Dairy Science Innovation at the University of Nottingham (UK) which has about 150 Holstein dairy cattle and 15 pre-weaned calves (in one barn at the time of the study); this was used for a pilot study (Chapter 3). The second farm was a local commercial farm (Leicestershire, UK) with about 200 mixed breed dairy cattle and about 70 pre-weaned calves.

2.3 Barns Assessment

Dimensions of the barns, doors, type and approximate quantity of bedding, ventilation, and calf pens were measured and recorded. For the full study (Chapter 4), wind speed, temperature and humidity were measured throughout the period of sampling by HoldPeak (HP-866B, UK), and EasyLog USB (Lascar Electronics,

UK), respectively. Moreover, type of feed, milk, and places of the troughs for water and feed were recorded.

2.4 Environmental assessment

Depending on the number of calves and size of the barn, three sites were chosen for each farm at evenly distributed locations inside the barn for both studies. Air from each of these sites was sampled to evaluate the concentration of bacteria and to allow identification of the three viruses.

2.4.1 Oxoid air sampler

Airborne bacterial samples were collected using a portable impaction-type Oxoid air sampler (Thermo Scientific, UK) at different locations inside the pen (the sampling locations are detailed in the appropriate chapters). The Oxoid air sampler works by aspirating the air through a grid perforated with a pattern of 219 calibrated holes. The resulting air streams containing microbial particles were directed onto an agar surface of microbiological media. The air sampler was positioned vertically and approximately 0.6m above the bedded area in all locations.

2.4.2 MD8 Airscan

Air samples for the detection of viral and bacterial nucleic acid were collected using an MD8 Airscan (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with presterilized gelatine filters (Sartorius) (Figure 2.1). The MD8 has a pump unit (size 300 mm x 175 mm x 210 mm; weight 7 kg) which was attached to a 1500 mm length, 38 mm diameter flexible plastic hose to a filter head which incorporates an 80 mm diameter gelatine membrane filter (pore size 3 μm). The MD8 was positioned vertically and approximately 0.6m above the bedded area in all locations

as described by Lago et al. (2006). The air was sampled onto a gelatine membrane filter for collecting total airborne bacterial and viral nucleic acid, and the sampler set to collect 800L of air in a 6-min period (flow rate 133 litres/min). Samples were kept in sterile bags at − 20°C until further processing whereupon the gelatine filter was divided in half for DNA and RNA extraction.

Figure 2.1: Air samplers for detection of both bacteria and viruses. (A) an MD8 air sampler for collecting bacteria and viruses through gelatine filter, (B) an Oxoid air sampler for collecting total bacteria directly onto bacterial plates.

2.4.3 Culture and identification of bacteria

After aspiration the air by Oxoid air sampler the air will be sampled onto a sheep blood agar (BA plates; Thermo Scientific™ Oxoid™ Blood Agar) for total bacterial counts with the sampler set to collect 10 litres of air (Figure 2.2 A). Sampling onto eosin methylene blue agar plates (EMB; Thermo Scientific™ Oxoid™ Eosin Methylene Blue Agar) for gram-negative bacterial counts used 25 litres of air

(Figure 2.2 B). Samples were collected in triplicate for all sites. The EMB plates were incubated at 37°C for 48 h and BA plates for 24 h and manual colony counts were performed. Total bacterial count was corrected using the instruction manual for the Oxoid air sampler and colony forming units per 1000 L of air (1m^3) were calculated. The maximum count detectable by the air sampler was 1307 CFU/m^3. Mean and standard deviation was calculated for each sample site and air volume in 1 m^3, using GraphPad Prism software, version 9.1.0.

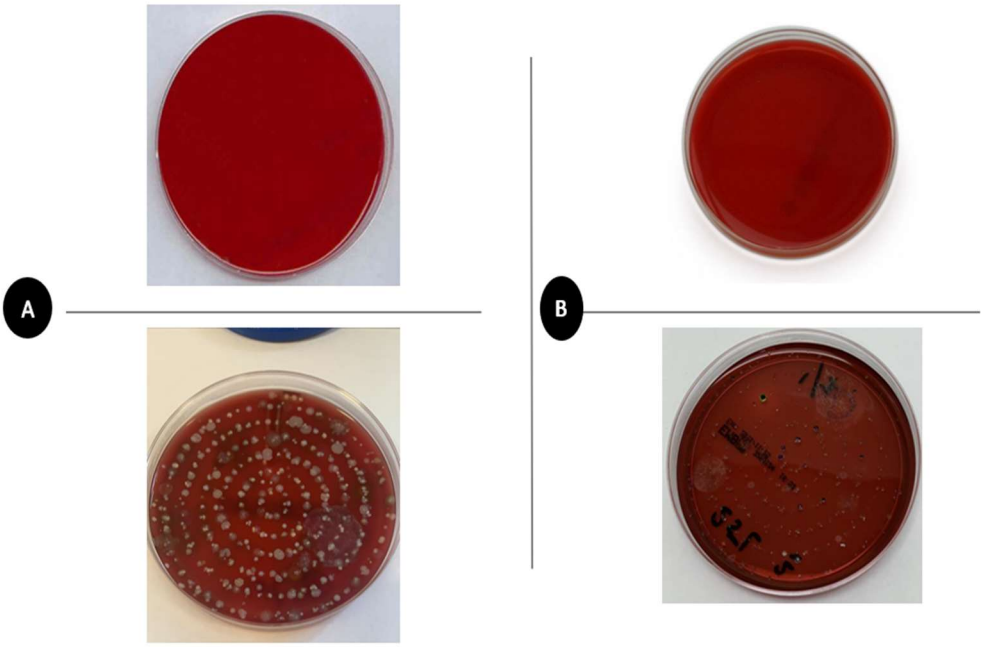

Figure 2.2: Bacterial plates for both gram-negative and positive bacteria.

The diagram shows the bacterial plates that were used in this study (A) BA plates pre- and post- sample collection (B) EMB plates before and after samples collection. After sample collection the BA plates were incubated at 37°C for 24 h, while EMB plates were incubated at 37°C for 48 h.

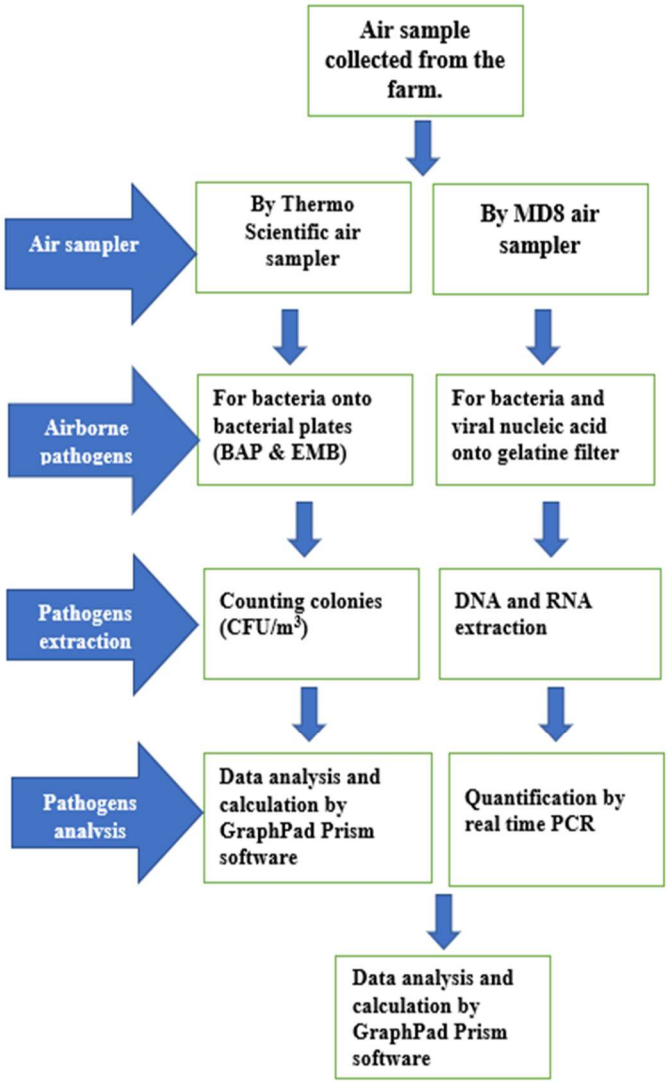

Figure 2.3: Flow diagram of sampling methods, procedure, and analysis for airborne bacteria and viruses.

2.5 DNA and RNA extraction and quantification

2.5.1 DNA extraction

Total DNA was extracted from a gelatine filter using QIAamp Fast DNA Stool Mini kit (QIAGEN, UK) following the manufacturer's instructions. Twenty gm of gelatine filter was placed into a 5 mL centrifuge tube, 2 ml of inhibitEX buffer was added to the gelatine, and the contents were mixed well by vortexing for 1 min. After thoroughly homogenised, the mixture was then incubated at 70 C for 5 min,

vortexed for 15 s, and then centrifuged for 1 min. Then, 15 µL of proteinase K and 200 µl supernatant were added into a new 1.5 ml centrifuge tube with 200 µL Buffer AL. The mixture was then incubated at 70◦C for 10 min. The tube was briefly centrifuged and 200 µl of ethanol (96 – 100%) was added to the sample and mixed by vortexing for 15 s. From this mixture, 600 µl was transferred to the high filter QIAamp spin column, and then centrifuged at 6000 xg for 1 min. The tube was placed into a clean 2 mL collection tube, and the tube containing the filtrate was discarded, then the above step was repeated. Then 500 µl of AW1 buffer was added to the QIAamp spin column, centrifuged for 1 min at 6000 x g, then the column was placed in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. Then 500 µl of buffer AW2 was added to the column and centrifuged at highest speed (20,000 xg) for 3 min to remove any residual wash buffers. The QIAamp spin column was inserted in a clean 1.5 mL microcentrifuge tube and the total DNA was eluted by adding 200 µl of buffer ATE, incubating at room temperature (15 – 25◦C) for 1 min, and then was centrifuged at 6000 xg for 1 min. Eluted total DNA samples were stored at –20˚C until further use.

2.5.2 Viral RNA extraction

Viral RNA was extracted using QIAamp® Viral RNA Mini Kit (Qiagen, UK) following the manufacturer's instructions. Twenty grams of gelatine filter was removed to a 5 mL centrifuge tube, 560 µl of prepared buffer AVL containing carrier RNA was added to the gelatine and the contents were mixed well by pulse vortexing for 15 s. The mixture was then incubated at room temperature (15 – 25◦C) for 10 min. The tube was briefly centrifuged, 560 µl of ethanol (96 – 100%) was added to the sample which was then mixed by pulse vortexing for 15 s. From this

mixture, 630 µl was transferred to the high filter QIAamp Mini column, which was then centrifuged at 6000 x g for 1 min. The tube was placed into a clean 2 mL collection tube, the tube containing the filtrate was discarded, and the above step repeated. Then 500 µl of AW1 buffer was added to the QIAamp Mini column, which was centrifuged for 1 min at 6000 xg. The column was then placed in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. Then 500 µl of buffer AW2 was added to the column and centrifuged at the highest speed (20,000 xg) for 3 min to remove any residual wash buffers. The QIAamp Mini column was then inserted in a clean 1.5 mL microcentrifuge tube and viral RNA was eluted by adding 60 µl of buffer AVE, and then was centrifuged at 6000 xg for 1 min. Eluted total RNA samples were stored at –20˚C until further use.

2.5.3 Detection of nucleic acids

2.5.3.1 Detection of DNA for total airborne bacteria and BoHV-1 by qPCR

Quantitative polymerase chain reaction (qPCR) was used to quantify virus BoHV-1and total airborne bacteria in gelatine filter samples as described before by Hay et al. (2016). Target species and genes for the airborne BoHV-1 and bacteria, probe, and primers were as detailed in Table 2.1. PCR was performed for DNA quantification in 25 ul volumes composed of master mix and other reagents (Table 2.2).

Twenty microliter of master mix and 5 µl of DNA sample were added to a specific place in the 96-well plate (alpha Laboratories, UK). The positive controls used for BoHV-1 were from the Bovilis® IBR marker vaccine (MSD Animal Health, UK) used to “spike” a gelatine filter. This was done by taking 1 µl from a live vaccine,

dropping it in clean gelatine filter, and then extracting DNA as detailed in 2.5.1. The negative control used a clean gelatine filter that was subject to the same extraction protocol. Five-fold serial dilutions were conducted and calculation the median tissue culture infecting dose per mL (TCID50/mL) for BoHV-1 with a starting concentration of 25500 TCID50/ml. Regarding the bacteria, the positive control was a kind gift from Dr Sharon Egan (The University of Nottingham, UK) which was prepared in a similar manner to BoHV-1 vaccine. The qPCR conditions and cycles for DNA were as follows: denaturation at 95°C for 2 min, annealing 55°C for 30 s, and final step at 74°C for 5 minutes for 45 cycles. All reactions were performed in duplicate using a Light Cycler® 480 (Roche). The results were analysed by using GraphPad Prism software, version 9.1.0.

Table 2.1: primer and probe sequence for airborne total bacteria and viruses.

Target species	Target gene	Primers and probes sequence (5'- 3')	Melting point	References
Total airborne bacteria	16s rRNA	747F- 50-TGGGGAKCAAACAGGATT-30 R- 50-GTAAGGTTCTTCGCGTTG-30 6FAM-ACCCTGGTAGTCCAYGCYGTAAAC-BHQ1	60.1 ˚C to 70 ˚C	(Lima et al. 2016)
BoHV-1	gB[#]	F- TGTGGACCTAAACCTCACGGT R- GTAGTCGAGCAGACCCGTGTC FAM AGG ACC GCG AGT TCT TGC CGC–BHQ	65.5˚C to 75.5˚C	(Thonur et al. 2012)

The table illustrates target species, genes, primers, probes, and melting points to detect and quantify total airborne bacteria and BoHV-1. All the primers and probes were obtained from (Sigma-Aldrich, UK), [#]Target gene: glycoprotein B (gb), F: forward and R: reverse.

Table 2.2: Components and volumes of qPCR for quantifying DNA for total airborne bacteria and BoHV-1.

Component	Volume/ µl
PCR grade water	2.12
5X GoTaq® Flexi Buffer (Promega)	12
Probe	0.25
MgCl2 Solution	2
dNTP Mix	0.5
Forward primer	1
Revers primer	1
GoTaq® MDx DNA Polymerase (colourless)	0.13
Template DNA	5

The table components, concentrations, and volumes of reagents for qPCR. MgCl2 solution containing magnesium chloride, and dNTPs mix containing deoxyribonucleotides, were supplied by Promega, UK.

2.5.3.2 Quantification of the RNA for viruses by Real-Time PCR

RT-PCR was used to quantify RNA viruses BRSV and PI3 in gelatine filter samples using a modification of the method described by Thonur et al. (2012). Target species and genes for RNA viruses, probes, and primers are shown in Table 2.3. PCR was performed using RNA extracted from gelation filters using a 20ul volume composed of master mix and other reagents given in Table 2.4.

Table 2.3: primer and dye sequence for RNA viruses.

Target species	Target gene	Primers and probes sequence (5' - 3')	Melting point	References
BRSV	N	F-GGTCAAACTAAATGACACTTTCAACAAG R-AGCATACCACACAACTTATTGAGATG 6FAM-TAGTACAGGTGACAA(+C)A(+T) (+T) G(BHQ1)	63.9 ˚C 73.2. ˚C	(Thonur et al. 2012)
PI3	M	F-TGTCTTCCACTAGATAGAGGGATAAAATT R-GCAATGATAACAATGCCATGGA 6FAM-TGCACAGCAATTGGATCAATAACT(+C) TAT(+T)C(BHQ1)	63.9 ˚C 73.2˚C	(Thonur et al. 2012)

The table shows target species, genes, primers, dye, and melting points to detect and quantify RNA viruses BRSV and PI3. All the primers were obtained from Sigma-Aldrich, UK, while SYBR Green (dsGreen) was from Lumiprobe, Germany. Target gene show nucleoprotein(N) and matrix(M). F: forward and R: reverse.

Table 2.4: Elements of qPCR for measuring RNA for BRSV, PI3.

Component	Volume/ µl
PCR grade water	3.6
Sensifast OneStep Mix	10
dsGreen	0.2
Forward primer	0.8
Revers primer	0.8
Reverse transcriptase	0.2
RNAse inhibitor	0.4
Template RNA	4

The table illustrates the components and volumes of reagents for qPCR. Sensifast OneStep Mix contains Magnesium Chloride (Mg), deoxyribonucleotide(dNTPs), stabilisers and enhancers, while dsGreen contains SYBR green. All the components are from (Meridian Bioscience, UK) except the primers and SYBR® Green from Sigma-Aldrich, UK and Lumiprobe, Germany, respectively.

Twenty microliter of master mix and 5 µl of RNA sample were added to a specific well in a white 96-well qPCR plate (Alpha Laboratories, UK). The positive controls for the viruses BRSV and PI3 were from the Rispoval® intranasal live vaccine (Zoetis, UK). Five-fold serial dilutions were conducted, and calculation the median tissue culture infecting dose per mL (TCID50/mL) for both viruses BRSV and PI3 starting at a concentration of 28000, 695000 TCID50/ml, respectively. The qPCR conditions and cycles for RNA viruses were as follows: reverse transcription at 45°C for 15 min, initial denaturation at 95°C for 2 min, followed by 45 cycles with denaturation at 95°C for 5 s followed by annealing and extension for 30s at 60°C. All reactions were performed in duplicate using a Light Cycler® 480 (Roche). The results were analysed by using GraphPad Prism software, version 9.1.0.

2.5.3.3 Identification of bacteria by Gram staining

Bacterial colonies from both EMB and BA plates were stained using Gram stain (Thermo Scientific, UK) to classify and identify bacteria (Bartholomew & Mittwer 1952) using the following procedure. First, one drop of free nucleic acid water (QIAGEN, UK) was added onto a clean slide, and one colonise from bacterial plate was taken and then mixed with water drop then left the slide until completely dry. The slide was then covered with crystal violet staining reagents (Thermo Scientific™, UK) for 1 min, and then the slide was gently washed with tap water for 2 sec. Then Gram's iodine was added to the slide for 1 min, and then the slide was gently washed with tap water for 2 sec. Then decolourising agent (95% Ethanol) was added for 15 sec, and then gently washed the slide with tap water for 2 sec. Then counterstain (Safranin) was added for 30 sec, and then gently washed the slide with tap water for 2 sec. Finally, the slide was left until completely dry and then observed the result under a brightfield microscope. The gram-positive bacteria appeared in blue/ purple colour, while the gram-negative appeared in pink/red stain.

Chapter 3: Pilot Study

3.1 Introduction

Any microorganism, including viruses and bacteria, can become airborne. Several techniques, including impactors, impingers, and filters have been applied for airborne viral and bacterial sampling (Verreault et al., 2008). Airborne bacteria and viruses can be detected by collecting air through air samplers onto a suitable medium, such as bacterial plates or gelatine filters, and then evaluating the media for the presence of the pathogen using a suitable assay (Zhao et al. 2014a). Many air sampling devices are available to collect airborne pathogens (bacteria and viruses), such as MD8 and Oxoid air sampler. MD8 air sampler has been used to collect airborne viruses and bacteria onto a gelatine filter (Reza et al. 2020). MD8 are generally considered more effective than other devices such as Andersen 6-stage impactor, all-glass impinger "AGI-30" and OMNI-3000 , and the physical efficiency may reach to 100% (Zhao et al. 2014b). The Oxoid air sampler for bacteria impacts the air directly onto an agar surface of microbiological media, so it is not suitable for collection of viruses (Reza et al. 2020, Kim et al. 2016). There is no recommended standard for air sampling volumes in the calf barns (Roque et al. 2016). Different air sampler devices such as impaction, impingement and filtration are likely to require different sample volumes. Additionally, farm factors such as the number of calves in barn, ventilation system, and pen size likely affect the optimal sampling volume (Zhao et al. 2014a). Therefore, some preliminary experiments were considered essential to ensure the validity of the methods used.

3.2 Aims and Objectives

Aim: To determine the optimum conditions for use of the air samplers for collecting airborne bacterial and viruses.

Objectives:

1. Determine optimum time for air sampling onto EMB and BA plates using the Oxoid air sampler.

2. Determine optimum time for incubation of bacterial plates prior to colony counting.

3. Collect samples onto gelatine filters for optimising nucleic acid extraction techniques.

3.3 Materials and methods

3.3.1 Study Sites and Calf Data

The study was conducted at the Centre for Dairy Science Innovation at the University of Nottingham (UK). A calf pen housing 14 Holstein and 1 mixed breed calves of mixed-sex aged approximately 2- to 3-month-old was chosen to obtain air samples in February 2021. All the 15 calves had been vaccinated by Rispoval intranasal live vaccine (Zoetis, UK) against two common viral diseases: bovine respiratory syncytial virus and bovine parainfluenza 3 virus. Some of the calves were diagnosed with BRD, with clinical signs including reduced feeding, depression, and cough about two weeks before sample collection. The calves had been treated before sample collection with meloxicam, anti-inflammatory and tulathromycin antibiotic for two days. At the day of sample collection, all the calves appeared healthy, and there were no clinical signs present in the animals except for

four of them which still had a mild cough (Table 3.1). Fifteen calves were housed in the naturally ventilated pen with a semi-open front. A positive ventilation tube ran along the underside of the roof and there was an open ridge in the roof. The pen size was approximately 11m x 5m, bedded with straw around 5 cm in-depth, with a passageway 5m x 1m in the rear area of the pen (Table 3.1). Calves were fed by an automatic calf feeder which was placed between the pen and passageway. Calves were drinking the water through an automatic water trough, and there was standing water in a drain around the feeder.

Table 3.1: Clinical signs and treatment for calf respiratory disease

Calf number	Clinical signs	Treatment
1	Stop feeding, depression, increase of respiratory rate and high temperature.	Meloxicam and Tulathromycin (for 2 days)
2*	Cough, nasal discharge, increase respiratory rate and stop feeding.	Meloxicam (for 2 days)
3*	Cough, nasal discharge, increase respiratory rate and stop feeding.	Meloxicam (for 2 days)
4*	Cough, nasal discharge, increase respiratory rate and stop feeding.	Meloxicam (for 2 days)
5*	Cough	Meloxicam (for 2 days)

* Still had a mild cough

3.3.2 Sampling procedure

3.3.2.1 Airborne bacterial collection using the Oxoid air sampler

The samples were collected at 10:30 am from the Centre for Dairy Science Innovation at the University of Nottingham (UK). Airborne bacterial samples were collected using a portable impaction-type Oxoid air sampler (Thermo Scientific, UK) at different locations inside the pen. The Oxoid air sampler aspirates air through a grid perforated with a pattern of 219 calibrated holes. The resulting air

streams containing microbial particles are directed directly onto an agar surface of microbiological media. The locations of samples were at mid-pen, in the right-hand corner by the concentrate trough, in the rear area of the pen, and by the outer door of the pen open to the fresh air (Figure **3.1**). The air sampler was positioned vertically and approximately 0.6m above the bedded area in each location. The air was sampled onto a sheep blood agar (BA plates) for total bacterial counts with the sampler set to collect 5, 10, 50 or 100 litres of air and eosin methylene blue agar (EMB plates) for gram-negative bacterial counts with 25, 50 or 100 litres of air. The EMB plates were incubated in an inverted position at 37°C for 48 h and BA plates for 24 h and kept overnight at 4C before colony counts were performed (CFU). Samples were collected in triplicate or quintuplet at each site.

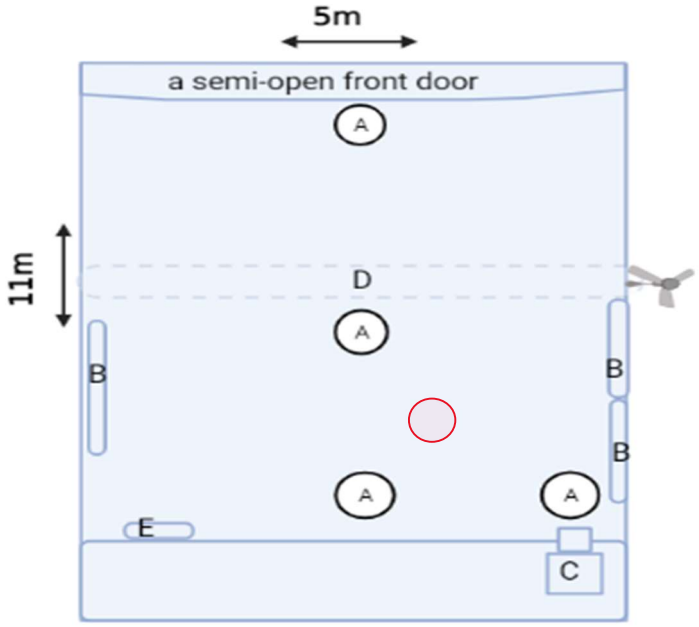

Figure 3.1:Dimensions of pen and sampling sites. The figure shows pen size and the sites of air sampling for the airborne bacteria (A), and the sampling site for viral/bacterial nucleic acids using the MD8 (red circle), the concentrate troughs (B), an automatic calf feeder (C), the positive ventilation tube in the roof with fan (D) and an automatic water trough (E).

3.3.2.2 Airborne viral and bacterial collections by MD8

Airborne viral and bacterial samples were collected using an MD8 Airscan (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with the presterilized gelatine filters (Sartorius). Air samples were collected during a 6-min period with flow rates were (133 litres/min). The total of the collected air was 800 litres. This was chosen to ensure a relatively high volume of air was sampled to maximise the potential yield of viral nucleic acid, while not having too long a period of sampling to minimise disruption to the calves. Samples were kept at − 20°C until further processing. Subsequently, each gelatine filter was divided into two halves for extracting both DNA for total bacteria and BoHV-1, and RNA for PI3 and BRSV. DNA and RNA nucleic acid were extracted (as described in 2.5).

3.3.3 Statistical analysis

All colonies forming units (CFU) were counted manually. Total bacterial count was corrected using the manufacturer guidelines of Oxoid air sampler for correction of total bacterial count. Colony forming units per 1000 L of air ($1m^3$) were calculated according to the equation below:

$$x = \frac{Prx1000}{V}$$

Where:

V= volume of sampled air

Pr= Probable count obtained by positive hole correction

x = Colony Forming Units per 1000 litres (= 1 cubic metre) of air

Mean and standard deviation were calculated for each sample site, using GraphPad Prism software, version 9.1.0. The data were compared using one–way ANOVA or student's *t* test as appropriate.

3.4 Results

3.4.1 Airborne bacterial counts on blood agar plates by Oxoid air sampler

The reasons for collecting different air amounts as well as places, in the first trial, were to optimise the air quantity and avoid saturation of agar plates from microorganisms. Due to the air sampler contains 219 calibrated holes, therefore, the maximum organisms count for the air sampler is 1307 CFU/m^3 (according to the equation provided in the manufacturer's instructions). This is the most probable number according to the manufacturer's instructions before the equation which is $\frac{Prx1000}{V}$. In the first experiment, air samples collected the total bacterial load was counted using BA plates with different air volumes (5, 10, 50, or 100 litres) at various places within the pen (middle, right corner, and the front door of the pen). Mean, standard deviation, minimum and maximum of microorganisms (as CFU/m^3) are presented in Table **3.2**. The mean of the bacterial counts on BA plates ranged from 2450 to 17467(SD) CFU/ m3. In the middle of the pen, the mean of the 5 litres was 17467 ± 12220 (SD) CFU/m3, while for 10 litres found to be 13000 ± 900 (SD) CFU/m3(Figure 3.2 B). For 50 and 100 litres the mean was 5980 ± 548 (SD) and 5537 ± 1006 (SD) CFU/m3, respectively. Furthermore, in the right corner of the pen, the mean for 50 litres was 5280 ± 824 (SD) CFU/m^3, while for 100 litres regarded as 2450 ± 669 (SD) CFU/m^3. Additionally, in the outer door of the pen, the mean of the 50 litres was 2450 ± 669 (SD) CFU/m^3. Ten litres were chosen for further assessment using BA plates as it was apparent (Figure 3.2 A) that 10 litres were the optimal amount of air before the system became saturated (i.e. colonies

were the maximum that the system was able to detect). Ten litres was chosen in preference to five litres because the deviance between samples was lower than the 5L air sample, where the standard deviation is rather high. The highest CFU/m^3 was found at the outer door, although the results were not statistically significantly different comparing to the different sites with 10 litres of air sampled (Figure 3.2B).

Table 3.2: Descriptive statistics for BA plates CFU/m3 in the middle, right corner, and outer door of the pen for different air volumes.

Parameter	Mean	SD	Minimum	Maximum
Middle pen 5 litres	17467	12220	6800	30800
10 litres	13000	900	12100	13900
50 litres	5980	548.1	5560	6600
100 litres	5537	1006	4390	6270
Right corner pen 50 litres	5280	824.9	4560	6180
100 litres	2450	669.1	2010	3220
Outer door pen 50 litres	9550	2899	7500	11600

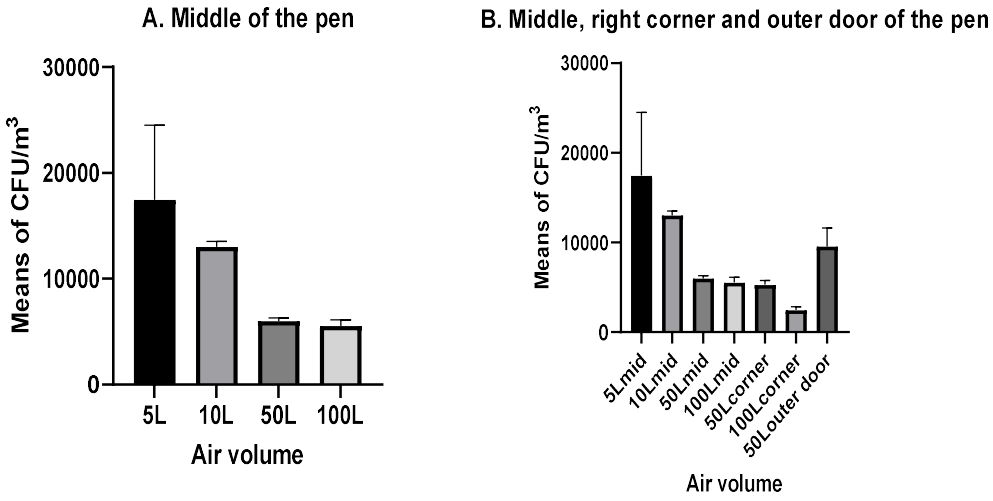

Figure 3.2: Comparison between the mean CFU/ m^3 on blood agar plates for different volumes of air (5, 10, 50 or 100). (A) at middle of the pen only. Bars show mean of CFU/ m^3 of samples and error bars the standard deviation and (B) at different sites (Middle, right corner, and outer door).

A second set of samples collected using 10 litres of air onto BA plates showed that there was no significant difference found between the rear area and the outer door of the pen (Figure 3.3).

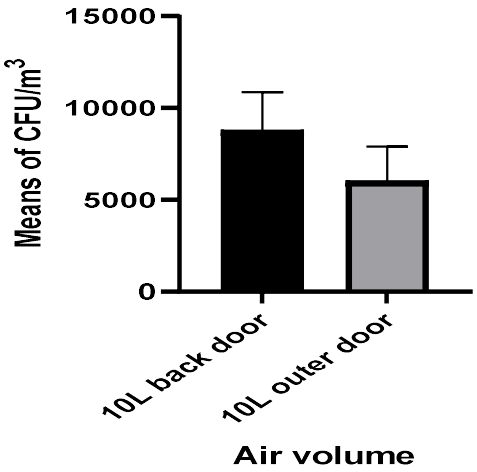

Figure 3.3: Bacterial counts for air samples (10L) collected onto blood agar plates, at two sites within the calf pen. Results did not show any significant difference found between the front and back area of the pen (p= 3, students t test). Bars show mean of (CFU/ m3) samples and error bars standard deviation.

3.4.2 Airborne bacterial counts on eosin methylene blue plates by Oxoid air sampler

The mean bacterial counts on EMB plates ranged from 960 ± 174 to 3707 ± 1709 (SD) CFU/m^3, depicted in Figure 3.4. At the front door of the pen, the highest counts were 3707 ± 1709 (SD) CFU/m^3, whilst in the back door of the pen, counts were 1087 ± 254 (SD) CFU/m^3. The three different volumes, 25, 50 and 100 litres in the back door of the pen, were fairly consistent with the mean counts ranging from 960 ± 174 to 1087 ± 254 (SD) CFU/m^3. The mean for the 25 litres sample was 960 ± 174 (SD) CFU/m^3, while for 50 and 100 litres the mean was 1087 ± 254 (SD) and 1047 ± 83 (SD) CFU/m^3, respectively. There is a significant different between the mean of the three different volumes of front door ($p>0.9$, one-way ANOVA test), with a higher count observed for the 25L sample. However, at the front door of the pen, the counts were inconsistent for the three different volumes (25, 50 and 100 litres) with the mean counts ranging from 1077 ± 303 to 3707 ± 1709 (SD) CFU/m^3. The mean of the 25 litres was 3707 ± 1709 (SD) CFU/m^3 while for 50 and 100 litres the mean was, respectively: 1633 ± 271 and 1077 ± 303 (SD) CFU/m^3. No significant difference found between the mean of the three different volumes of the back door ($p>0.9$, one-way ANOVA test).

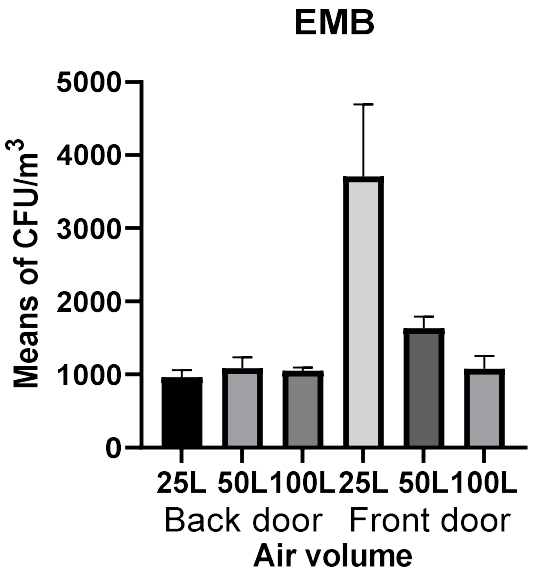

Figure 3.4: Mean counts of gram-negative airborne bacteria isolated in the EMB plates, different volume (25, 50 or 100L) at two different sites (back door and front door) in the calf barn. No significant difference found between the mean of the three different volumes of the back door ($p>0.05$), while there are significant different between the mean of the three different volumes of front door. Bars show mean of (CFU/ m^3) samples and error bars standard deviation.

3.4.3 qPCR optimisation results for bacteria and viruses

PCR optimisation was performed using positive and negative controls to identify the viruses (BoHV-1, BRSV, PI3) and total bacteria. Target species, genes, primers, and probes for both bacteria and viruses are described in section 2.5.3. Positive and negative controls performed as expected in the PCR assays.

3.5 Discussion

Since there is no recognised recommended standard for air sampling in animal farms, we performed a pilot study to determine optimal air volumes for sampling. Results revealed that 10 and 25 litres of air into BA and EMB plates, respectively,

seem to be the optimal air volumes for sampling using the Oxoid air sampler because the system was not saturated using these volumes. However, many factors play a role in the quantity of air volume required to collect airborne pathogens, such as choice of air sampler device, barn, temperature, ventilation, and the density of pathogens in the animal farms (Zhao et al. 2014a).

This study is consistent with previous research that used 10 litres of air inside and outside calf barns (Matković et al. 2007, Popescu et al., 2011). There are some differences between air samplers: their machine contains 300 holes, whereas our machine has 219 holes, and a different season between this study and that of Popescu et al. (2011). They demonstrated that 10L of air onto Columbia agar, Chapman agar, Endo agar, Blood agar, and Sabouraud agar was optimal for analysing the bacterial plates based on their preliminary study. It is possible that 10 L was optimal air volume in their study because they were dealing with adult cows, or the barns were relatively clean during the sampling. Hence, initial observations suggest that there may be no recommended standard for air sampling from calf farms – rather that each study should be optimised for local factors as mentioned above. However, several studies used different air volumes in calf barns, whether lower or higher than that of this study, and they have found many airborne bacterial pathogens present inside calf barns. For example, a field study by Lago et al. (2006) collected 5, and 10 litres of air by a machine contains 286 holes into BA and EMB plates, respectively, from 13 naturally ventilated calf barns. They found significant differences in bacterial counts between individual calf pens and alley in the same barn. This means even at the same barn, the density of pathogens may be different from site to site, which might be related to the cleanliness of the given area or the health status of calves in the immediate area. Additionally, Karwowska (2005) and

Zucker et al. (2000) measured the airborne bacteria in modern and old animal houses using different air volumes ranging from 50 to 250 litres. They found there was no significant difference between modern and old animal houses. Roque et al. (2016) showed that there is no recommended standard for air sampling volume using bacterial plates for collecting air samples from porcine, chicken, or bovine farms. However, to the best of the author knowledge, no report has been found so far using an Oxoid air sampler with flow rate 10L and 25L of air into BA and EMB plates, respectively.

In conclusion, we determined that the optimal air volumes for collecting total bacteria inside the barns by Oxoid air sampler into BA plates and EMB plates were 10 and 25 litres, respectively. The results obtained with these volumes in this study, using replicate samples were relatively consistent with low variance, however at one site (front door) there was greater variability between replicates. This might have been due to a gust of wind or movement of calves within the pen leading to higher counts in some of the replicates.

Chapter 4: Longitudinal Farm study

4.1 Introduction

Microorganisms, including viruses and bacteria, are present everywhere, and calf barns usually contain a variable but significant pathogen load. Bacterial and viral pathogens can contaminate the environment, and this contamination is considered a source of respiratory disease infection in calves (Poulsen and McGuirk 2009; Verreault et al., 2008). The number of pathogens inside the barn depends on different factors, such as the size of the barn, type of house, number of calves, health status, bedding area, and the ventilation system which, in turn, play an essential role in determining the temperature and humidity inside the barn (Cambra-López et al. 2010; Lang et al., 1997; Popescu et al., 2011).

This longitudinal study thus intends to further knowledge about airborne pathogens inside the calf barn and to assess these airborne pathogens to compared them with environmental factors. This longitudinal approach provides rich data by following the same farm over time and compared them with environmental factors to detect the causative agents that associated with BRD in the same barn. Pre-weaned calves are usually reared in a naturally ventilated barn that has individual pens, hutches and group housed (Lange et al., 1997). Despite recommendations for using hutches as the preferred housing for calves, many farm owners still use calf barns (Poulsen and McGuirk 2009, Lago et al. 2006). Poor ventilation results in increased temperature and humidity inside calf barns, and that may increase the number of microorganisms (Lago et al., 2006). Moreover, poor air quality is frequently responsible for developing stress in the calves, leaving them more vulnerable to respiratory diseases (Caswell 2014). Therefore, BRD is commonly associated with poor ventilation because the number of bacterial and viral pathogens would be increased inside the calf barns (Callan and Garry 2002). Airborne bacterial and

viral pathogens inside calf barns may be detected by air sampler devices to determine the pathogen load in the environment (Zhao et al. 2014b, Girlando 2014). This study used an MD8 sampler to detect airborne viral and bacterial pathogens using gelatine filters and an Oxoid air sampler to detect total airborne bacteria by sampling directly onto bacterial plates. This study was conducted to find out the causative agents for BRD and whether these causative agents are affected by the environmental factors such as temperature, humidity, calf number and age of calf. It was hypothesised that calf aged 6 weeks or under are equally susceptible to the BRD compared with those aged over 6 weeks.

4.2 Aims and objectives

The aim of this chapter was to study air contamination in a typical UK calf barn, specifically:

1- To isolate total airborne bacteria inside the calf barn and determine CFU counts using an Oxoid air sampler.

2- To collect total airborne bacteria and viruses using an MD8 sampler onto a gelatine filter to allow detection and quantification by (RT)-PCR of BoHV-1, and RNA for PI3 and BRSV and bacterial nucleic acid.

4.3 Materials and methods

4.3.1 Study sites and calf data

The study was performed at a local commercial farm (Leicestershire, UK) with about 200 mixed breed dairy cattle and about 80 pre-weaned calves. The calf barn was housing, on average, 64 mixed breed calves of mixed-sex aged from one week

to 14 weeks during the study. After the age of 12-14 weeks, the calves were fully weaned and removed from the barn. The pre-weaned calves were reared in a naturally ventilated barn with three opened doors, the barn contained about 19 pens, and each pen housed either 10 or 5 calves. However, at the beginning of the study, only 9 pens were used with a total of 41 calves. The barn size was approximately 35m x 15m, bedded with straw around 5 cm in-depth. Two temperature and humidity sensors were placed inside the barn throughout the study (Figure 4.2). Calves were fed pelleted feed ad libitum and fed twice daily with replacement milk by a manual calf feeder. Also, drinking water was provided by an automatic water trough that was placed in each pen. This barn was chosen to obtain air samples in May and June because this was the time for cows' parturition at this farm and a single visit was conducted weekly with a total of 7 weeks of sampling.

The total numbers of calves for each week were counted, and their age was recorded. Veterinarians examined each calf in each sampling week using the Wisconsin Calf Respiratory Scoring System (University of Wisconsin-Madison). The Wisconsin Calf Respiratory Scoring System was based on rectal temperature, character of nasal discharge, ocular discharge and presence of cough (Table 4.1).

Table 4.1: Wisconsin healthy scoring system for calf respiratory disease complex

	Scoring point			
Clinical sign	0	1	2	3
Rectal temperature (°F)	(100–100.9)	(101–101.9)	(102–102.9)	(>103.0)
Nasal discharge	Normal serous	Small amount of unilateral, cloudy	Bilateral, cloudy, or excessive mucus	Severe bilateral, mucopurulent nasal discharge
Eye discharge	Normal clear	Mild ocular discharge	Bilateral purulent ocular discharge	Severe bilateral purulent ocular discharge
Cough	None	Induce single	Induce repeated	Repeated spontaneous coughing

Total scoring 4 watch, and 5 indicated an urgent need for treatment

The vaccination and treatment record were collected from farm records at the end of the study. All the calves had been vaccinated by Rispoval intranasal live vaccine (Zoetis, UK) against two common viral diseases: bovine respiratory syncytial virus and bovine parainfluenza 3 virus. During and prior to the study, some of the calves were diagnosed with BRD, with clinical signs including reduced feeding, high temperature, depression, nasal and ocular discharge and cough. The calves were treated for two days with anti-inflammatory Metacam (meloxicam), antibiotics Draxxin (tulathromycin) and Resflor (florfenicol/flunixin) and anticoccidial Tolracol (toltrazuril) as shown in Figure 4.1.

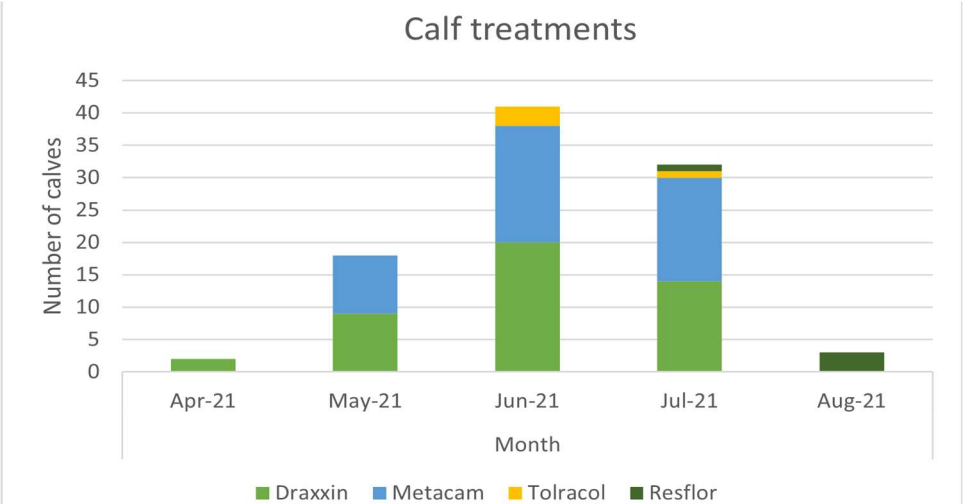

Figure 4.1: Treatment records for the sick calves before, during and after the sampling period. A low number of calves being treated in April and at the beginning of May is probably because there were few calves in the barn at one time. Most of the calves would have been weaned during late July and August, and the barn was empty.

4.3.2 Sampling procedure

4.3.2.1 Airborne bacterial collection using an Oxoid air sampler

Airborne bacteria samples were collected in triplicate using an Oxoid air sampler at three sites inside the barn for 7 weeks (Figure 4.2). The air sampler was positioned vertically and approximately 0.6m above the bedded area in each location. The air was sampled onto a sheep blood agar (BA plates) for total bacterial counts with the sampler set to collect 10 litres of air and eosin methylene blue agar (EMB plates) for gram-negative bacterial counts with 25 litres of air. The BA plates were incubated in an inverted position at 37°C for 24 h and EMB plates for 48 h and colony counts were then performed (CFU).

Figure 4.2: Dimensions of barn and sampling sites. The figure shows barn size (35 × 15m) and pen numbers (white circles), barn doors (white squares), and the sampling site for airborne bacteria using the Oxoid air samplers, and for viral/bacterial nucleic acids using the MD8 for all weeks except in week 6 and 7 where 3A changed to 3B (red circles). Site of temperature and humidity sensors (green rectangle).

4.3.2.2 Airborne viral and bacterial collections by MD8

An MD8 air sampler was used to collect airborne viral and bacterial samples once at each site 1, 2 and 3(A/B) inside the barn for 7 weeks (Figure 4.1). Air samples were taken for a 6-minute period at a flow rate of 133 litres/min. The air was sampled onto a presterilized gelatine filter, and then each gelatine filter was divided into two halves for extracting both DNA to detect total bacteria and BoHV-1, and RNA for PI3 and BRSV detection. DNA and RNA nucleic acid were extracted and quantified as described in sections 2.5 and 2.5.3.

4.3.3 Statistical analysis

For the purposes of this research, descriptive and observational study designs were employed, with the prevalence of airborne microorganisms evaluated in relation to the specific environmental factors. Airborne bacterial samples were collected in triplicate by an Oxoid air sampler and samples for DNA/RNA extraction once each week by MD8, and environmental parameters were collected during all weeks of sampling. All colony forming units (CFU) were counted manually. Total bacterial count was corrected using the manufacturer's guidelines. Colony-forming units per 1000 L of air ($1m^3$) were calculated according to the equation below:

$$x = \frac{Prx1000}{V}$$

Where:

V= volume of sampled air

Pr= Probable count obtained by positive hole correction (from manufacturers guidelines).

x = Colony Forming Units per 1000 litres (= 1 cubic metre) of air

Mean and standard deviation were calculated for each sample site, using GraphPad Prism software, version 9.1.0. The data for environmental pathogens were correlated with environment parameters (barn temperature, humidity, calf number and calf age). The data were compared using one–way ANOVA, student's *t* test and Pearson r correlation test as appropriate.

4.4 Results

4.4.1 Overview of data obtained

Mean, standard deviation, minimum and maximum for barn and calf data over the duration of the entire sampling period are presented in Table 4.2. Mean temperature, relative humidity and wind speed inside the barn were found to be 17.35°C ± 1.67 (SD), 72.1% ± 5.90 (SD) and 0.35 m/min ± 0.19 (SD) respectively. This study was used two temperature sensors in the middle and right corner of the pen and the data from the right corner sensors were discarded because the temperature was approaching 40°C, which suggests that the sensor was exposed either to direct sunlight, or the wall may be heated up during the day. The mean number of calves present at each time of sampling was 64.5 ± 13.20 (SD), and the total number of calves for each week is presented in Figure 4.3, while the mean calf age was found to be 6.47 ± 2.22 (SD) weeks. The mean pen number was 13.14 ± 2.34 (SD), and the total pens in use each week is presented in Figure 4.4. Table 4.3 shows the temperature and relative humidity inside the barn during all weeks of sampling. The highest mean barn temperature was 20°C in week 5, while the lowest mean barn temperature was 14.9°C in week 1. The highest barn relative humidity was 78.3% in week 7, while the lowest was 63% in week 3.

Table 4.2: Descriptive statistics of the calf, barn, and environmental data from the naturally ventilated barn during all weeks of study.

Parameter	Mean	SD	Minimum	Maximum
Calves, no	64.57	13.20	41	80
Calf age, wk	6.47	2.22	1	12
Nasal discharge score	13.57	13.60	2	39
Ocular discharge score	17	9.8	3	33
Cough score	3.57	1.6	2	6
Calf temperature score	101.6	0.85	99.5	105.2
Pens, number in use	13.14	2.34	9	15
Temperature inside the barn (°C)	17.35	1.67	7.5	29.5
Humidity inside the barn (%)	72.1	5.90	30.5	95.5
Wind speed (m/min)	0.35	0.19	0.1	0.7

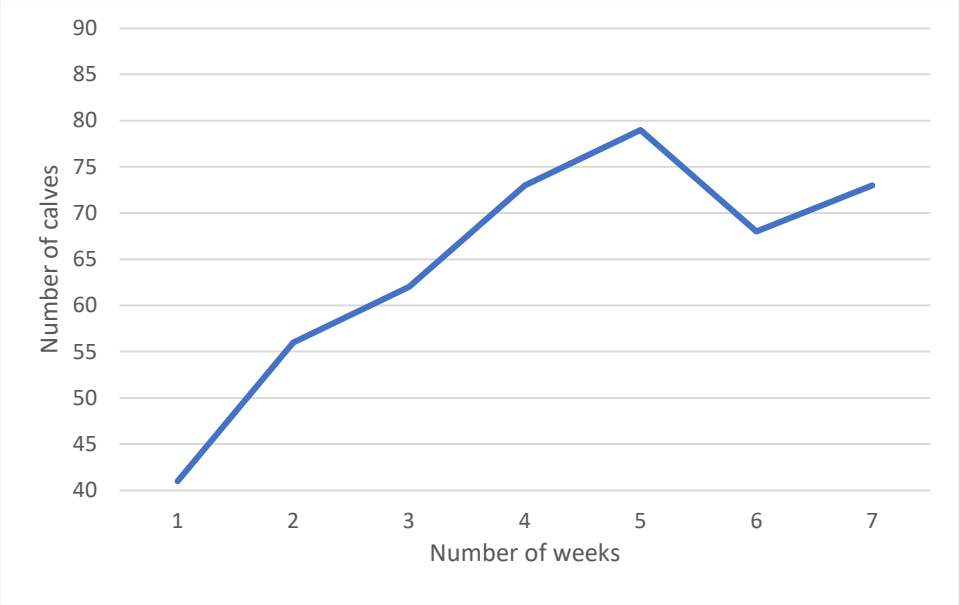

Figure 4.3: The number of calves for each week of sampling. The minimum number of calves was 40 at week 1, and the maximum was 79 calves at week 5.

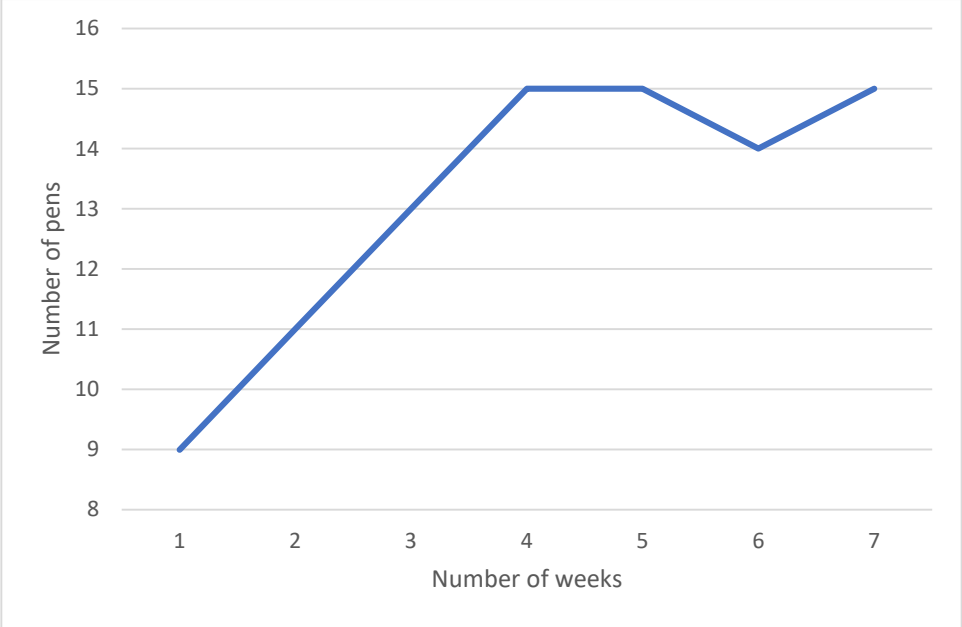

Figure 4.4: The number of pens for each week of sampling. The minimum number of pens was 9 at week 1, and the maximum was 15 at weeks 4,5 and 7.

Table 4.3: Barn temperature and humidity for each week during the sampling (middle of barn).

Number of weeks	**Wk 1**	**Wk 2**	**Wk 3**	**Wk 4**	**Wk 5**	**Wk 6**	**Wk 7**
Temperature of the barn (°C)							
Minimum	7.5	9.5	11.5	8	9	9	13.5
Maximum	25	27.5	29.5	28	25	25	26.5
Mean	14.9	18.6	20	16.8	16.1	17.2	17.9
Humidity of the barn (RH)							
Minimum	35.5	34	34.5	30.5	32.5	36.5	51
Maximum	91	87.5	85	95.5	93	93.5	93.5
Mean	68.8	66.8	63	74.1	76.8	76.9	78.3

4.4.2 Calf scoring

The mean of scoring of calf respiratory disease for nasal discharge, ocular discharge, cough and calf temperature over the duration of the entire sampling period was presented in Table 4.2. In addition, the proportion of clinical scores for calf respiratory disease during all weeks of sampling is shown in Figure 4.5. The most frequent clinical finding was an elevated rectal temperature (>101°F; 60%), and the lowest was coughing (4%), whereas the ocular discharge and nasal discharge scores were found in 20% and 16% of calves, respectively.

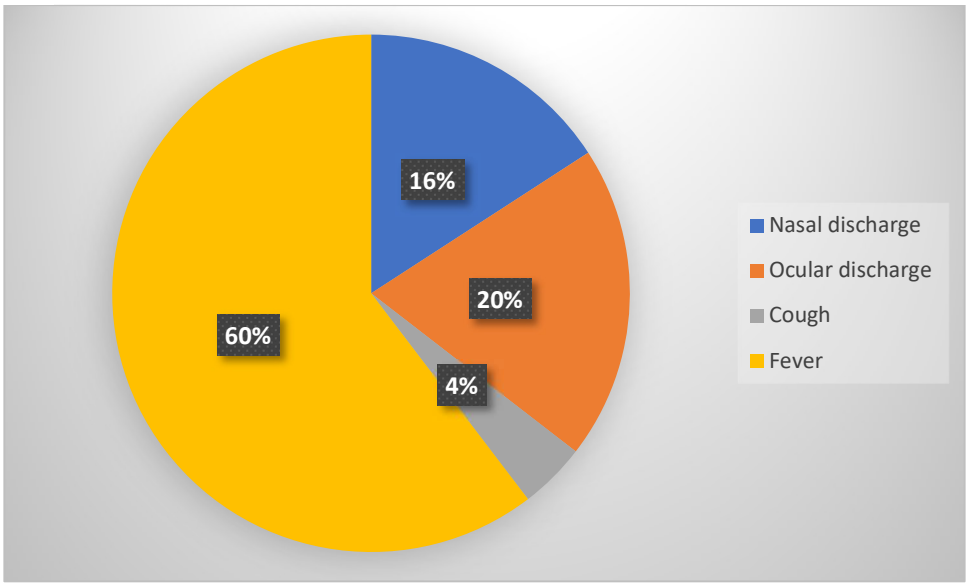

Figure 4.5: Proportion of each clinical scores for calf respiratory disease during all weeks of sampling.

4.4.2.1 Calf respiratory scores for each week

Scoring for each week of study was divided into those calves which were visibly healthy (calves score = 0) and rectal temperature ≤100.9°F, versus calves showing any clinical signs (those with any clinical signs, and rectal temperature, ≥101°F). Therefore, the number of visibly calves showing any clinical signs was higher than

healthy calves over the sample period. The highest proportion of calves showing clinical signs was seen at week 5, while the highest proportion of healthy calves was at week 4. There was a statistically significant difference between the number of healthy and calves showing clinical signs (**** p=0.0001, a student's paired *t-test*) (Figure 4.6 A). On the other hand, the mean of calves' temperature score for each week (Figure 4.6 B) shows a different variation between each week except weeks 2 and 4 varies much less than other weeks. The highest individual calves' temperature was at week 5 (105.2 °F) and the lowest at week 2 (99.5 °F). The mean calf temperature in week 1 (102 °F ± 0.94, SD) and week 5 (101.8°F ± 0.98 SD), were significantly greater that in week 6 (101.2°F ± 0.77 SD), respectively **** p= 0.0001 and **** p= 0.0001, one-way ANOVA test. Furthermore, the mean of calf temperature in week 3 (101.8°F ± 0.75 SD) and week 7 (101.8 °F ± 0.90 SD) were significantly greater that in week 6 (101.2°F ± 0.77 SD), respectively (***p= 0.0003, ***p=0.0004, one-way ANOVA test; Figure 4.6 B). The calves' temperature for all weeks was tested for possible correlation with barn factors (temperature, humidity, total calf number and bacterial counts on BA and EMB plates), however there was no relationship between calf temperature and barn factors (P>0.5 two tailed, Pearson r correlation test).

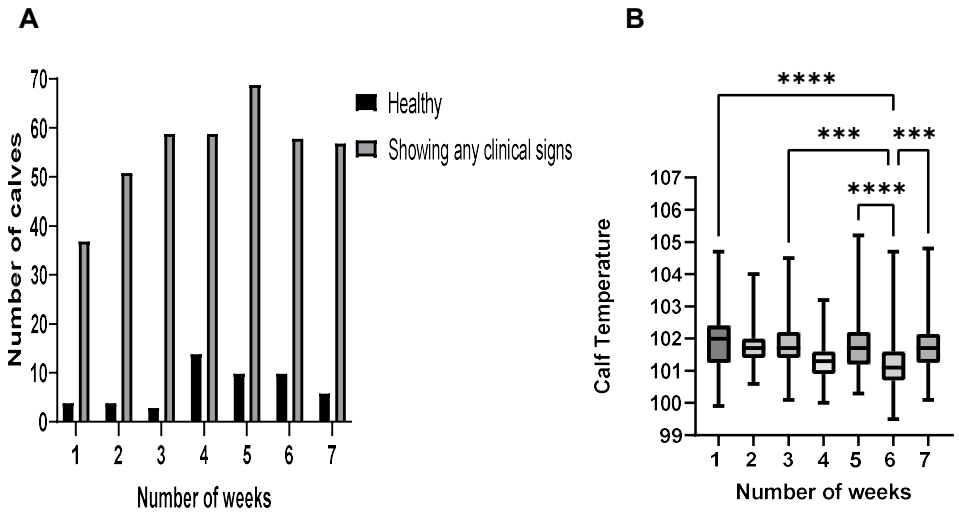

Figure 4.6: Health scoring system for calf respiratory disease based on visible signs. (A) shows the number of healthy calves with score – 0, versus calves showing any clinical signs including (nasal discharge, ocular discharge, cough and temperature) (score > 0) and temperature score (≥101°F). (B) Boxplot representing the mean of calf temperature for each week. Lower and upper horizontal lines of the box represent 1st quartile (Q1) and 3rd quartile (Q3), respectively. Horizontal line within the box indicates the median of calf temperature (Q2). The lower/upper whisker extends from the Q1/Q3 represent the minimum and maximum calf temperature. (**** p= 0.0001 and ***p= 0.0003).

4.4.2.2 Prevalence of calf respiratory disease with age

The calves ages range between 1 to 14 weeks (Figure 4.7). The boxplot in 4.7 suggests that the barn filled with progressively younger calves as the period of weeks increased because the new-born calves were housed in the barn. In order to simplify data analysis, the data was classified into two groups: 6 weeks or under and greater than 6 weeks. Assessed in this way, 328 out of 451 (73%) calves were aged 6 weeks or under and 123 out of 451 (27%) aged over 6 weeks during all weeks of sampling. The null hypothesis was that calf aged 6 weeks or under are equally susceptible to the BRD compared with those aged over 6 weeks. This

hypothesis was not confirmed as the results of this test showed that the calves aged 6 weeks or under are significantly more susceptible to BRD than calves aged over 6 weeks (p= 0.028; Table 4.4). In addition, Figure 4.8 also showing the total scoring of calves for each week of age. It is clear from this figure the higher total scores are in calves of 6 weeks or less of age than calves aged over 6 weeks.

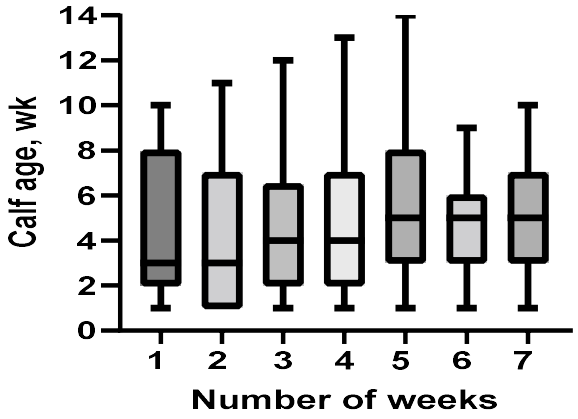

Figure 4.7: Boxplot representing the calf age for each week. Lower and upper horizontal lines of the box represent 1st quartile (Q1) and 3rd quartile (Q3), respectively. Horizontal line within the box indicates the median of calf age (Q2). The lower/upper whisker extends from the Q1/Q3 represent the minimum (1 week) and maximum (14weeks) of calf age.

Table 4.4: Relationship between calf scoring for ocular, nasal signs, cough and temperature with calf age.

Age	Healthy	Clinical Signs	Total
<6 weeks	30	298	328
>6 weeks	21	102	123
Total	51	400	451
Fisher's exact test	The two-tailed P = 0.028		

Healthy calf showing Wisconsin Score = 0 in any category and temperature (≤100.9°F), compared to calves showing Wisconsin Score > 0 in any category and temperature (≥101°F).

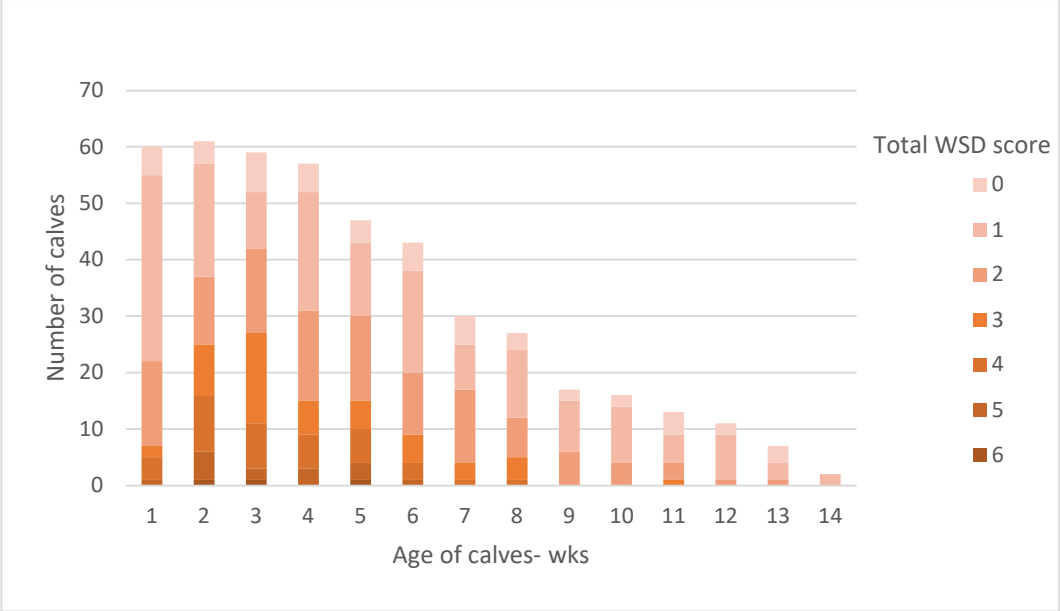

Figure 4.8: Total scoring of calves for each week of age. The figure shows the total scoring > 0 in any category, coughing, ocular or nasal signs and fever for each week of age (Score = 0 calves not showing any signs).

4.4.3 Airborne bacterial sampling

As shown in Figure 4.9, the bacterial counts from air samples were quite variable from week to week or depending on the site of sampling. The mean of the CFU/m^3 counts on BA plates for all sites over the duration of the entire sampling period was 18,219 ± 11,676 (SD) CFU/m^3. The majority of bacterial counts were in the range 10,000 – 20,000 CFU/m^3. The highest BA plates bacterial count was observed at site 1 in week 7 (58,833 CFU/m^3) and the lowest one at site 3 in week 4 (5,200 CFU/m^3). No significant difference found between the mean colony counts at the three different sites for BA plates (p=0.30 one-way ANOVA test; Figure 4.9 A, B and C). The mean colony counts on EMB plates for all sites over the duration of the entire sampling period was 2,013 ± 1,111(SD) CFU/m^3. The highest BA plates bacterial count was observed at site 1 in week 1 (4,987 CFU/m^3) and the lowest one at site 1 in week 4 (480 CFU/m^3). No significant difference found between the mean

colony counts of the three different sites onto EMB plates (p=0.8 one-way ANOVA test; Figure 4.9 D, E and F).

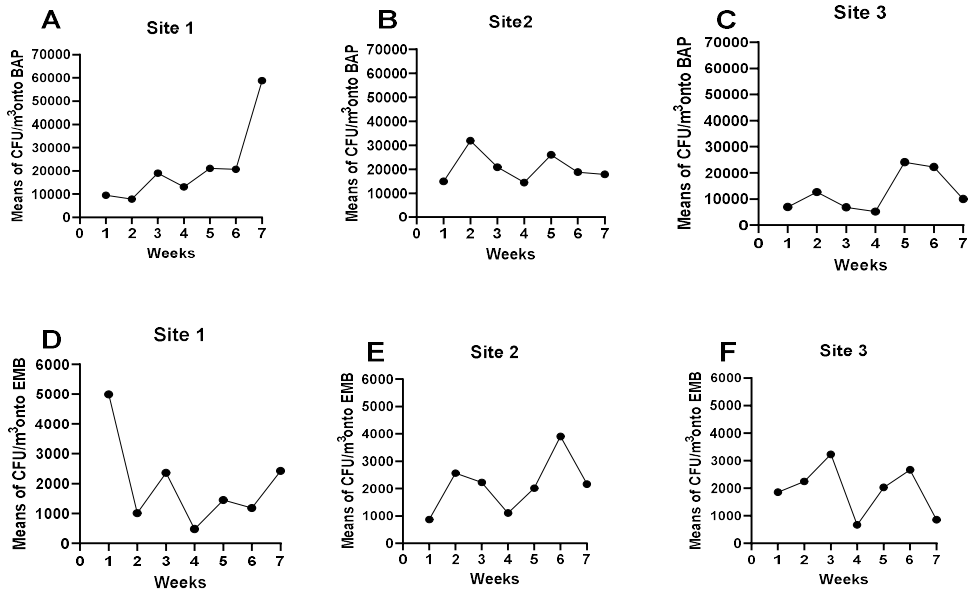

Figure 4.9: Mean of CFU/ m^3 in all sites (1,2 and 3) onto BA and EMB plates.

Comparison between the mean CFU/ m^3 on blood agar plates for different sites (1,2 and 3) (A), and (B) between the mean CFU/ m^3 on eosin methylene blue plates for different sites (1,2 and 3). The line graph show means of CFU/ m^3 of samples. Site 1 has the greatest variation and at week 7 there are the highest counts on the BA plates, but for site 1, the counts are highest on the EMB plates at week 1 when the counts on the BAP are low.

4.4.3.1 Airborne bacterial counts on blood agar plates and correlation with barn temperature, humidity, and calves' number

After air sampling onto blood agar plates and incubation, mean total bacterial counts for each different site (1,2 and 3) were determined (CFU/m^3) and correlated with the mean of the temperature, humidity, and calves number (Figure 4.10 A, B and C). Barn temperature and humidity were measured for all weeks of sampling and presented (Table 4.3). The mean of barn temperature, relative humidity and calf number for seven weeks of sampling were 17.35°C ± 1.67 (SD), 72.1% ± 5.90 (SD) and 64.57 ± 13.20 (SD), respectively. Therefore, the mean CFU/m^3 of total airborne

bacteria onto BA plates was correlated with those factors (Temperature, humidity, and calves' number), and there was no statistically significant relationship between the mean of CFU/m^3 on BA plates of all sites with temperature, humidity, and calves' number respectively, (P= 0.69, P=0.19 and P=0.27, two tailed, Pearson r correlation test).

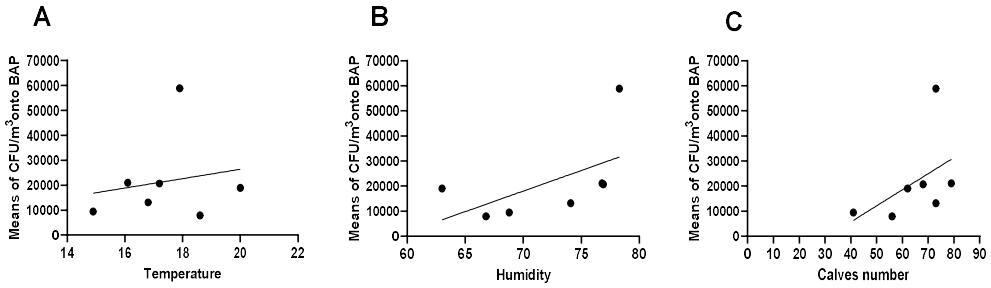

Figure 4.10: Correlation between the mean CFU/ m^3 of all sites during 7 weeks on blood agar plates with temperature (A), humidity (B), and number of calves (C).

4.4.3.2 Airborne bacterial counts onto eosin methylene blue plates and correlation with barn temperature, humidity, and calf numbers

Following air collection onto eosin methylene blue plates and incubation, mean of total bacterial counts (CFU/m^3) were obtained and correlated with the means of temperature, humidity, and calves' number (Figure 4.11 A, B and C). The barn data shows that the mean of temperature, humidity (Table 4.3) and calf number for the seven week study were 17.35°C ± 1.67, 72.1% ± 5.90 and 64.57 ± 13.20 (SD), respectively. Mean of temperature, humidity and calves' number were correlated with the mean of CFU/m3 on EMB plates. There was no relationship between the mean of CFU/m3 on EMB plates and barn temperature, barn humidity and calves' number respectively, (P= 0.39, P=0.54 and P=0.07, two tailed, Pearson r correlation test).

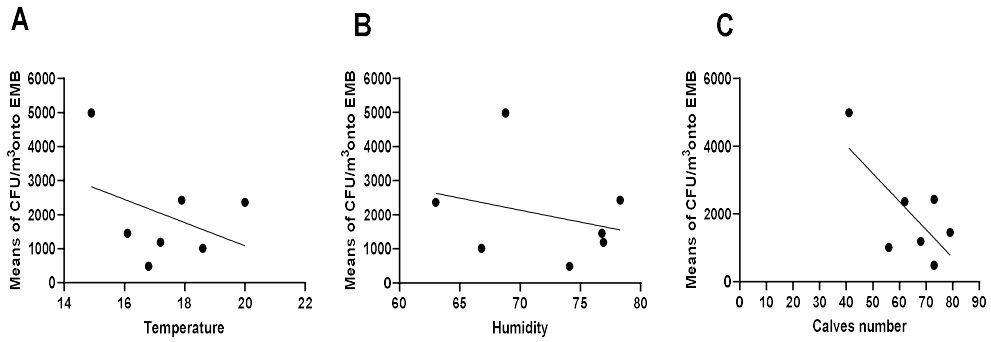

Figure 4.11: Correlation between the mean CFU/ m^3 of all sites during 7 weeks on eosin methylene blue plates with temperature (A), humidity (B), and number of calves (C).

4.4.4 Detection of airborne viruses by qPCR

A total of 9 out of 21 air samples were tested positive for PI3 during the 7 week study at three sites inside the barn. The number of PI3 copies was calculated using the obtained standard curve (Figure 4.12). Furthermore, a total of 3 out of 21 samples were tested positive for BRSV at some point during the study, and the number of BRSV copies was calculated using the obtained standard curve (Figure 4.13). The efficiency of the qPCR assay for PI3 was 2.406, while for BRSV was 2.185. Table 4.5 shows the summary of results for PCR detection of the viruses BoHV-1, PI3 and BRSV in air samples by qPCR It is apparent from this table that the PI3 was positive for all weeks except week 5 and present in all sites (1,2 and 3) at some point during the study. At weeks 6 and 7 in sites 1 and 2 PI3 was present as weak positive (Ct= 38; 12 TCID50 equivalent copies /33 litres of air; Figure 4.14). BRSV was detected only in weeks 5 and 7 at sites 1 and 3, respectively (Figure 4.15), while BoHV-1 was negative during all seven weeks of sampling (Figure 4.16). PI3 was detected more frequently at site two and least at site 1. Conversely, BRSV was detected more frequently at site 1 and least at site 3.

The three viruses (BoHV-1, PI3 and BRSV) were tested for possible correlation with the barn factors (temperature, humidity, calf number, health status of the calves), and no correlation was identified ($p>0.5$, two tailed, Pearson r correlation test).

Regarding the viral load in the positive air samples, PI3 was ranged between 408 to 70 TCID50 equivalent copies /33litres of air, while the BRSV ranged between 0.36 to 0.015 TCID50 equivalent copies /33 litres of air. The highest number of TCID50 equivalent copies /33 litres of air for PI3 was detected in the first four weeks of sampling at site 2 (408 TCID50 equivalent copies /33 litres of air), while the highest number of copies for BRSV was detected at week 5 at site 1 (0.36 TCID50 equivalent copies /33 litres of air). BoHV-1 was not detected at any site during all weeks of sampling.

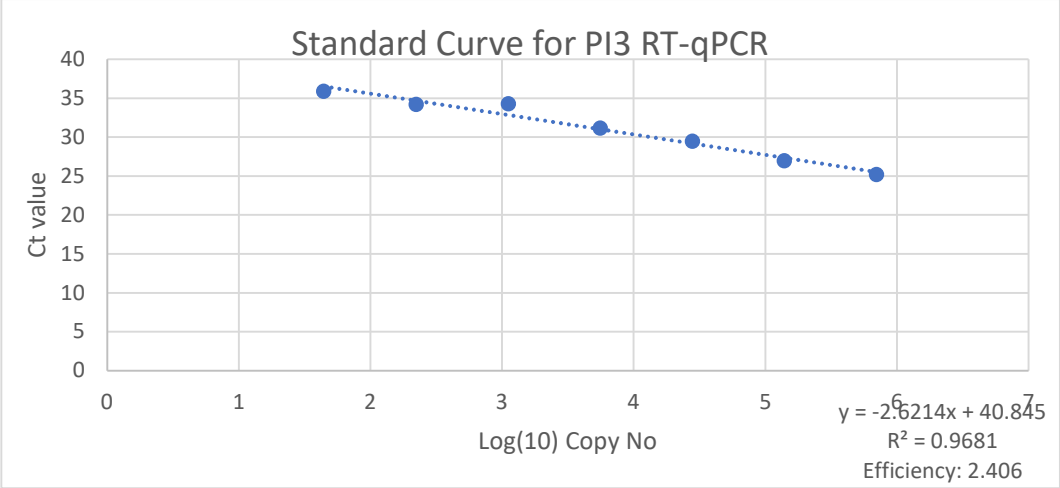

Figure 4.12: Standard curve for the calculation of PI3 copy number. X axis represents PI3 copy number and Y axis the measured Ct value.

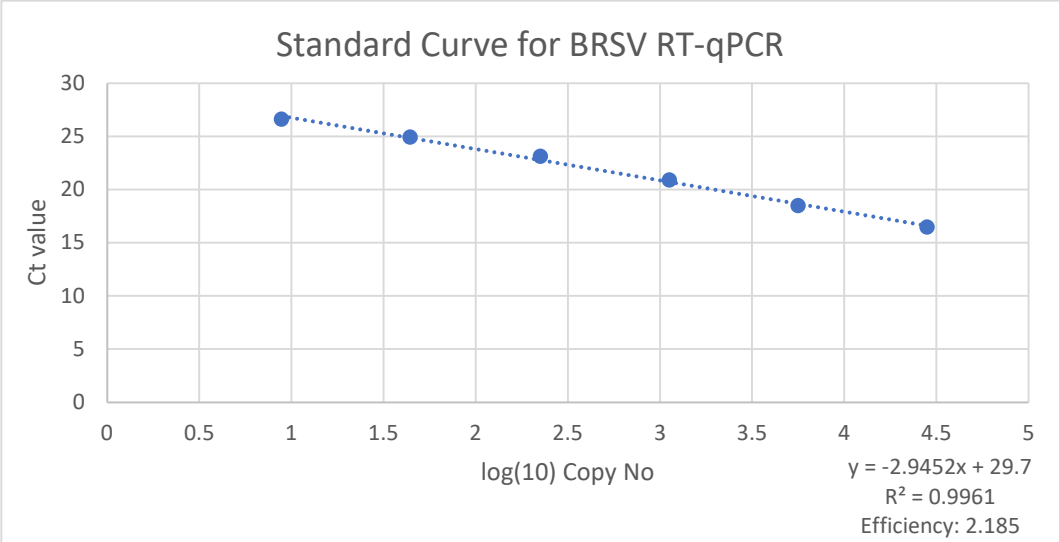

Figure 4.13: Standard curve for the calculation of BRSV copy number. X axis represents BRSV copy number and Y axis the measured Ct value.

Table 4.5: qPCR and RT-qPCR for detection of BRD viruses (BoHV-1, PI3 and BRSV) in air samples during seven weeks of sampling

Group site	Group week	Detection of viruses		
		BoHV-1	PI3	BRSV
1	1			
	2			
	3			
	4			
	5			
	6			
	7			
2	1			
	2			
	3			
	4			
	5			
	6			
	7			
3	1			
	2			
	3			
	4			
	5			
	6			
	7			

Sample analysis was performed in duplicate. Green colour indicates the virus was negative in qPCR, while red colour was positive. Yellow colour indicates the virus was weak positive Ct= 38; 12 TCID50 equivalent copies /33litres of air.

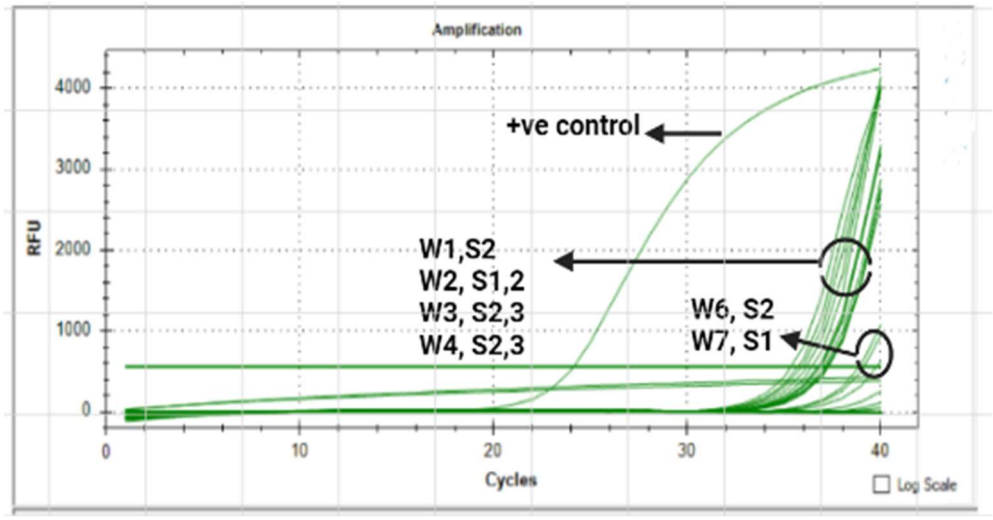

Figure 4.14: Amplification of PI3 by qPCR for all sites and weeks.

All samples were performed in duplicate. Figure shows the Ct values of positive control(+ve) and positive results for all weeks(W) and all sites(S) of sampling. The PI3 was negative in week 5

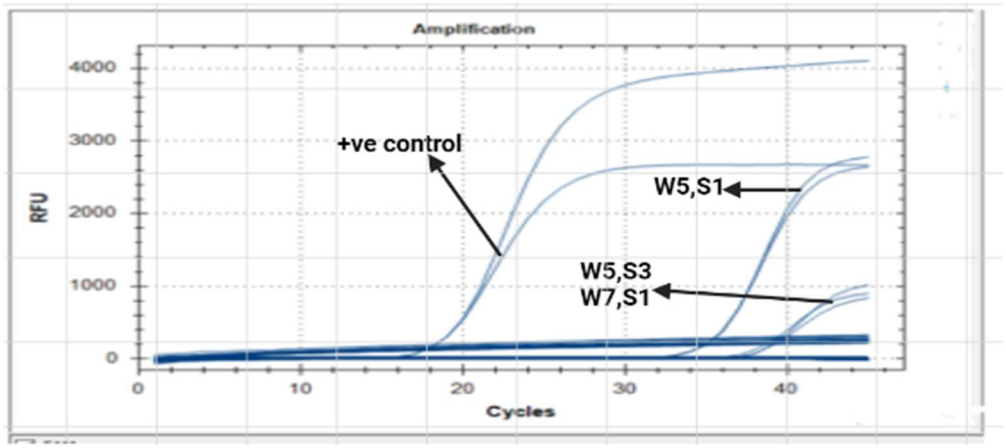

Figure 4.15: Amplification of BRSV by qPCR for all sites and weeks.

Figure shows the Ct value of positive control and positive samples for all weeks (W) and sites(S). BRSV was positive only at weeks 5 and 7 in sites 1 and 3.

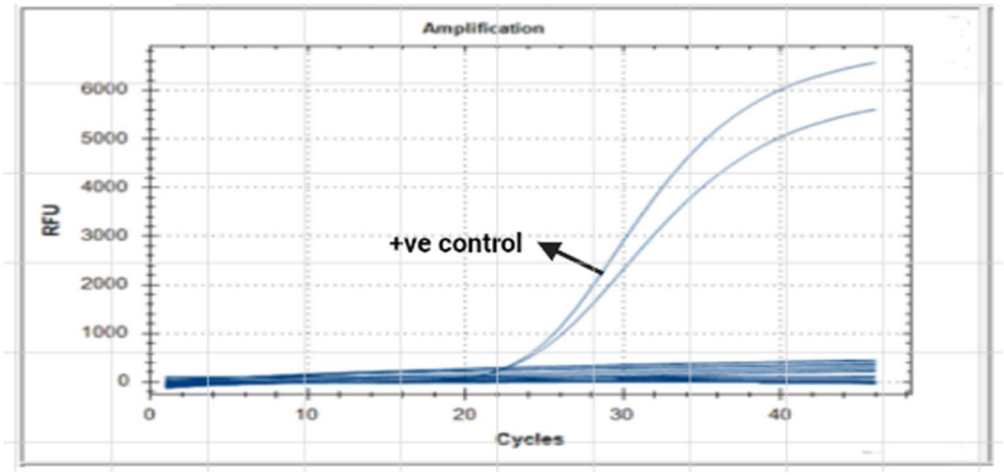

Figure 4.16: Amplification of BoHV-1 by qPCR for all sites and weeks.

The figure shows the positive control (+ve) for BoHV-1. No BoHV-1 was detected at all weeks of sampling.

4.4.5 Detection and quantification of airborne bacteria by PCR

Table 4.6 shows the summary of qPCR results for detection of total bacterial DNA (16s). As can be seen from the table (below), the Ct value was higher (++) in site 2 (mid-barn) for all weeks except week 6, while the lowest Ct value (+) was present more often at site 1. Figure 4.8 also shows the Ct value of total bacteria that were obtained from qPCR. During the first 4 weeks of sampling, the Ct value was 30 in all sites (1,2 and 3), while in the last 3 weeks was 35 (+) at week 5 and 6 sites 1 and 2, respectively. There was no statistical difference between each week and site ($p=0.8$ one-way ANOVA test). The total airborne bacteria were correlated with the barn factors (Barn temperature, barn humidity, calves' number, healthy status of the calves), and there was no relationship between total bacteria and barn factors ($p>0.5$, two tailed, Pearson r correlation test).

Table 4.6: qPCR for detection of total bacteria (16s) during seven weeks of sampling

Group site	Group week	Ct value of total bacteria
Site1	Week1	++
	Week2	++
	Week3	++
	Week4	++
	Week5	+
	Week6	++
	Week7	++
Site2	Week1	++
	Week2	++
	Week3	++
	Week4	++
	Week5	++
	Week6	+
	Week7	++
Site3	Week1	++
	Week2	++
	Week3	++
	Week4	++
	Week5	++
	Week6	++
	Week7	++

+ = Ct value ~35

++ = Ct value ~30

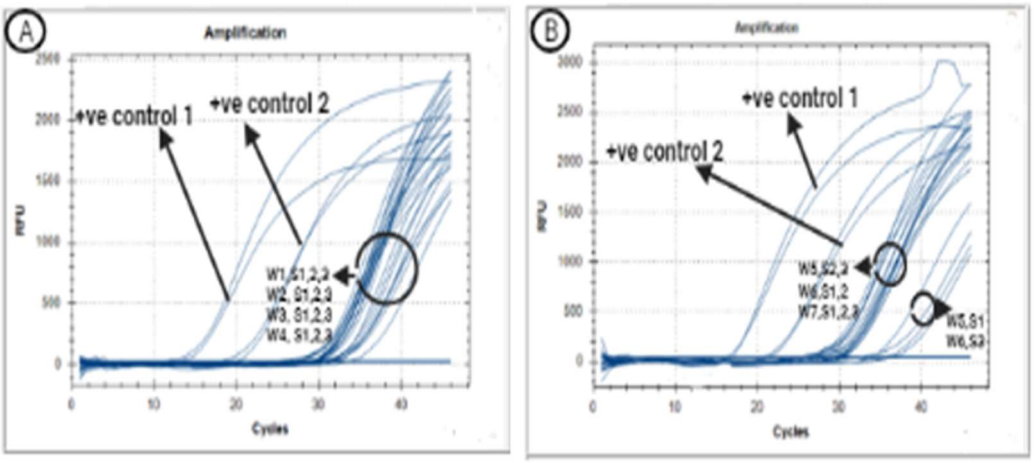

Figure 4.17: Amplification of total bacteria (16s) by qPCR for all sites and weeks. Both (A and B) show the positive control 1 (E. coli) and 2 (Staphylococcus), and (A) samples for all sites (1,2 and 3) and weeks 1,2,3 and 4, the Ct value during these weeks was 30. (B) shows sites (1,2 and 3) and weeks 5,6 and 7, the Ct value was 30 for all samples except week 5 sites 1 and week 6 site 2 were 35. Samples for weeks 1 to 4 were analysed on a separate plate from samples for weeks 5 to 7.

4.4.6 Classification of cultured bacteria by Gram staining

Samples of bacterial colonies from BA and EMB plates were cultured and stained by Gram stain. Figure 4.18 shows the colony shape that was observed from three representative colonies from BA plates sampled at sites 1, 2 and 3 within the barn. From this figure, we can see that the shape of cocci and purple colour which indicate gram-positive bacteria. Figure 4.19 shows the morphology of three colonies that were smeared from EMB plates sampled at sites 1, 2 and 3 within the barn. This figure shows the bacteria to have a rod shape and the red colour that indicates gram-negative bacteria.

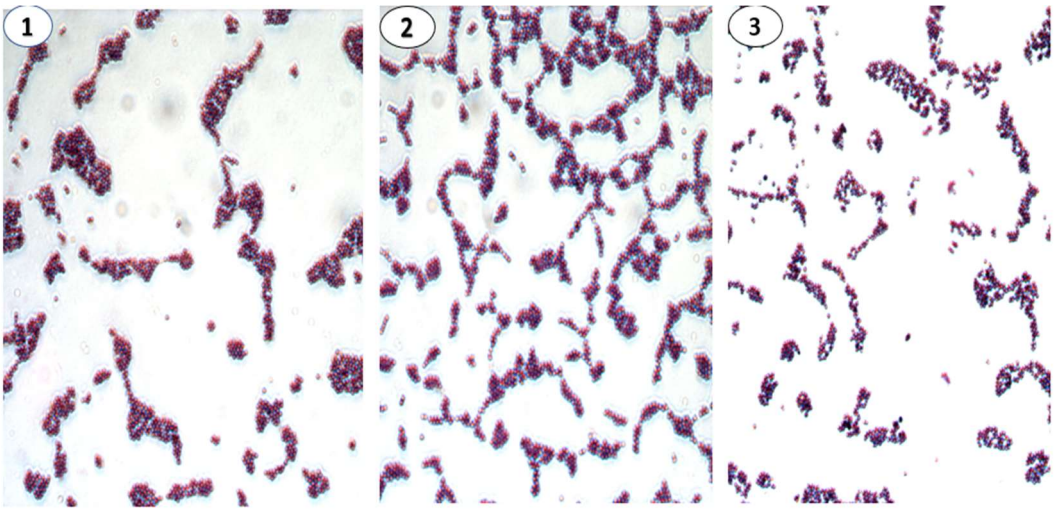

Figure 4.18: Gram staining of BA plates colonies from sites 1, 2 and 3 within the barn. (1) at site 1, (2) site 2, and (3) at site 3. The purple colour and cocci shape indicate to the gram-positive bacteria.

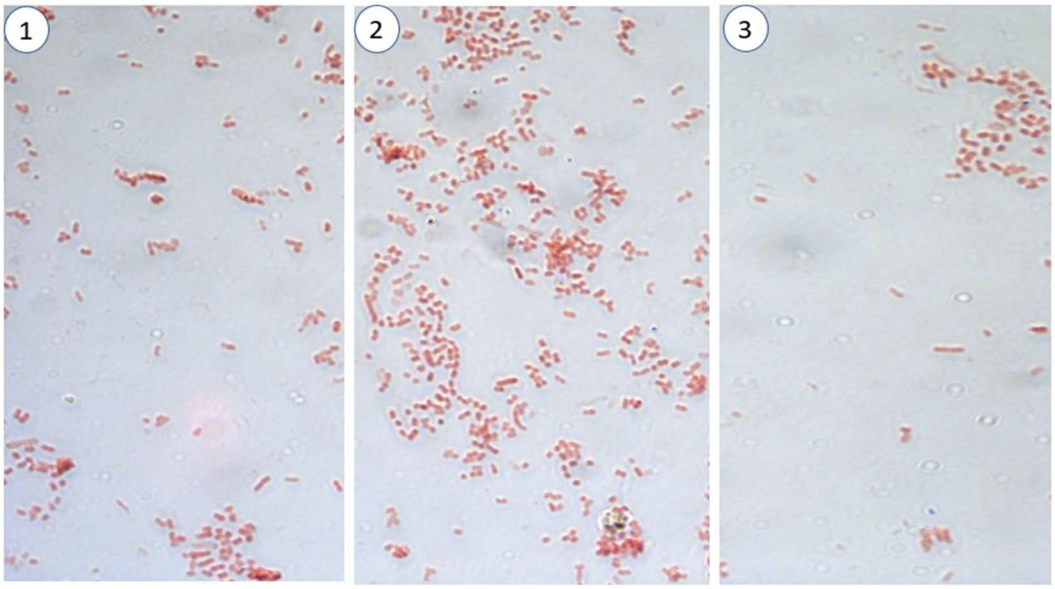

Figure 4.19: Gram staining for EMB colonies from sites 1, 2 and 3 within the barn. (1) at site 1, (2) site 2, and (3) at site 3. The red colour and rod shape indicate to the Gram-negative bacteria.

4.5 Discussion

Poor air quality frequently causes stress among calves, making them more susceptible to respiratory diseases (Caswell 2014). As a result of the increased number of bacterial and viral pathogens inside of calf barns, BRD is frequently correlated with poor ventilation (Gershwin et al., 2015). In this research, the total number of airborne bacteria as CFU per cubic metre (CFU/m^3) inside of a naturally ventilated barn were studied using an Oxoid air sampler. Moreover, nucleic acid from the total airborne bacteria and from three viruses, i.e. BoHV-1, BPI3 and BRSV, associated with BRD were collected using an MD8 air sampler to detect and quantify viral nucleic acid using qPCR. Three different sites inside of the barn were chosen for seven weeks, and barn conditions such as temperature and relative humidity were studied and recorded.

The health status of each calf was determined in each sampling week using the Wisconsin Calf Respiratory Scoring System. Based on the scoring results, the calves were treated with either antibiotics or anti-inflammatory drugs. Most animals treated for BRD respond immediately after initial BRD treatment due to the influence of antibiotics and anti-inflammatory drugs. However, some animals develop chronic BRD that is resistant to additional antimicrobial, ancillary or supportive therapy (Booker and Lubbers 2020). In this study, a low number of calves were being treated in April and at the beginning of May, probably because there were few calves in the barn at the time, and in late July and August, probably because most of the calves would have been weaned and the barn would have been empty.

This study showed that the calves aged six weeks or below were more susceptible to BRD than were the calves aged six weeks or above based on clinical scores. These results are consistent with other research on calf respiratory disease that reported a peak incidence of BRD at five and six weeks of age (Waltner-Toews et al., 1986), but slightly differ from those of Lago et al. (2006), who reported a peak incidence of BRD at seven weeks of age. This difference might be related to the vaccination schemes and the influence of antibiotics that were used for treating diseased calves in this study, or (alternatively) due to differences in calf genetics or housing methods.

The average temperature inside of the barn during all weeks of sampling was 17.35°C (range 7.5 to 29.5°C). A newborn calf's thermoneutral zone is between 10 and 26°C, while a one-month-old calf's thermoneutral zone is between 0 and 23°C (Wathes et al., 1984; Manzoor et al. 2019). Clearly, the young calves in this study that were below one month of age were exposed to temperatures below/above the thermoneutral zone, while one-month-old calves were exposed to temperatures above their thermoneutral zone for many weeks during the sampling period. It is possible that the temperature inside of the barn was stressful to the calves, which, in turn, may have affected their health status.

The impact of temperature upon calf farms depends on both the season and the location. In general, the diverse microclimatic circumstances in each locality have an important effect in determining the microbiological population; these microbial communities are exceedingly complex and responsive to seasonal variations (Quintana et al. 2020). Previous studies observed differences in the total number of airborne pathogens in the environment, depending on the season or due to meteorological factors (Vissers et al. 2007; Dungan et al. 2011). The results

revealed no correlation between the barn temperature and humidity and the airborne bacteria counts on BA and EMB plates. However, this study was limited to one particular farm during one period of seven weeks. Dungan et al. (2011) found that there was a negative correlation between the number of microorganisms and relative humidity. Furthermore, a study conducted in laboratory conditions found that increasing the air temperature reduced the bacterial survival time, resulting in lower bacterial counts (Wathes et al. 1984). In contrast, Lago et al. (2006) found that the airborne pathogens collected on BA plates increased with an increasing barn temperature. The observed difference could be because they conducted their study in winter and the temperature ranged from -6.7 to 12.2°C, or it could be because the density of calves affected the total number of airborne pathogens. In this current study, two temperature and humidity sensors were used inside of the barn at two different sites (middle and right corner of barn). The data from the right-corner sensors were discarded because the temperature was approaching 40°C, which suggests either that the sensor was exposed to direct sunlight or that the wall may have been heated up during the day. It is quite difficult to analyse the wealth of information present in 24-hour continuous environmental records, and more advanced analysis might be needed to take advantage of the data that we collected from the barn, e.g. using machine learning techniques. To address this issue, future studies should focus on using a larger trial that exploits airborne pathogens within different environments, seasons and regions, or using many environmental sensors inside of the barn to minimise any bias.

Total bacterial counts from air samples on BA and EMB plates were quite variable from week to week or depending on the sampling site, and the number of gram-positive bacteria on BA plates was higher than that of gram-negative bacteria on

EMB plates during all weeks of sampling. The average number of airborne bacteria on BA plates during all weeks of sampling was 18,219 CFU/m^3, which was not consistent with any previous study. It is possible that different results were observed because of the difference in air sampler devices (impaction, impingement, cyclone forces, or filtration) or the sampling site. All of the three sites inside of the barn showed different airborne pathogen numbers during all weeks of sampling, but there was no significant difference between the three sites. It could be because this study was conducted in one barn (with one large, open, airy space) or because the environmental conditions (temperature and humidity) were variable at each time point during the sampling. In this context, Lago et al. (2006) found significant differences in bacterial counts between individual calf pens and alleys in the same barn. Pathogen concentrations might differ from one site to the next, which might be related to the cleanliness of the given area or the health status of calves in the immediate area.

Airborne viral and bacterial samples were collected using an MD8 air sampler, utilising gelatine filters for the detection both of DNA for the total bacteria and BoHV-1 and of RNA for PI3 and BRSV. Understanding the mechanisms of viral and bacterial transmission is critical for developing effective control methods and preventing zoonotic diseases. Previous research on the environmental detection of pathogens has been conducted on (DNA and RNA) viruses that affect other animal species, including calves, such as myxoma virus (López-Lorenzo et al. 2021), Aleutian mink disease virus (AMDV) (Prieto et al. 2014, 2017), porcine circovirus type 2 (PCV2) in swine (Prost et al. 2019; Roque et al. 2016; Corzo et al. 2013), and avian influenza A (Blachere et al. 2018). The results showed that PI3 and BRSV RNA could be detected inside of the calf barn for several weeks, while BoHV-1

was negative during all weeks of sampling. To our knowledge, these are the first results regarding the environmental detection of BRSV and PI3 inside of calf barns. In this study, the animals in the barn had been vaccinated against PI3 and BRSV, but not against BoHV-1. Therefore, it is possible that the viruses were detected in the barn because the calves shed the vaccine strain. To address this issue, future studies should focus on using viral sequencing to differentiate between the field and vaccine strains.

Regarding the viral load in the positive air samples, PI3 ranged from 408 to 70 TCID50 equivalent copies/33 litres of air, while BRSV ranged from 0.36 to 0.015 TCID50 equivalent copies/33 litres of air. However, the limit of detection for PI3 was 70 TCID50 equivalent copies/33 litres of air at Ct 36, while for BRSV it was 0.015 copies/33 litres of air at Ct 35. The sensitivity of the assay would be enhanced by increasing the sampling time in order to collect a larger volume of air. In this way, a study conducted by López-Lorenzo et al. (2021) used an MD8 air sampler to filter 1,500 L of air for 30 min for the detection of DNA virus in a rabbit barn, which found high viral loads present on the farm as well as in items that were not in direct contact with the animals. Regarding the bacterial load in qPCR, our bacterial positive control concentration was unknown; therefore, it is not possible to comment on the detection limits for the assay. However, the results demonstrated that the Ct value for all total bacteria samples during all weeks of sampling was 30 Ct (except in weeks 5 and 6 at sites 1 and 2, respectively). Therefore, it would be interesting in future studies to measure the limit of detection for the total bacteria in the air by means of qPCR. In addition, supporting our findings via the collection of upper respiratory tract swabs from pre-weaning calves would be beneficial in determining and confirming the bacteria and viruses associated with BRD by means

of qPCR. As the bacterial PCR used was a non-specific one capable of identifying many bacterial species, it may serve as a general indication of “environmental cleanliness”; however, assays for specific bacteria for BRD may show a different relationship with calves or environmental factors.

In summary, this is the first study in the UK conducted on environmental contamination with PI3 and BRSV in calf barns in which vaccination schemes against these viruses are being carried out. PI3 and BRSV were consistently detected in air samples from the farm, while BoHV-1 was negative during the study. Furthermore, we determined the number of airborne pathogens for gram-positive and gram-negative bacteria. The results showed that the burden of gram-positive bacteria was higher than that of gram-negative bacteria, and none of the bacteria were affected by barn factors such as temperature, humidity, and the number of calves. Moreover, we found that the younger calves below the age of six weeks were more susceptible to BRD than were those above the age of six weeks.

Chapter 5: General Discussion

5. General discussion

Airborne microorganisms are considered to be one of the most serious issues in livestock production (Caswell 2014). Airborne pathogens and dust production are cattle farms are mostly caused by dry, warm weather and by active cattle behaviour (Verreault et al. 2008). BRD is considered to be one of the multifactorial diseases caused by environmental factors, including the density of airborne pathogens (viruses and bacteria), temperature, humidity, ventilation, concentrations of noxious gases, and particles of dust (Grissett et al., 2015). There are many airborne bacteria and viruses associated with BRD, and the optimal non-invasive way of detecting these pathogens is to collect air from calf barns using air samplers. Air sampling is increasingly recognised as an important tool for characterising and quantifying microorganisms in the air (Reza et al. 2020). Several techniques, including impaction, impingement, cyclone forces, or filtration, have been applied for airborne viral and bacterial sampling (Verreault et al. 2008).

Air sample collection can be affected by a number of factors such as location selection, time of sampling, sample volume, and air sampler type. Therefore, several additional parameters must be considered. Firstly, animals' behaviour plays a role in the concentration of microorganisms in a barn. For example, while the dust is inert, the microorganisms are alive and, therefore, must be collected in a way that preserves their viability to avoid minimising their concentration (Zhao et al. 2014a). Secondly, determining the optimal air volumes and sampling times for collecting airborne pathogens is vital in order to avoid device saturation, which would reflect the microbial counts. Thirdly, the interpretation of the microbial count results may differ depending on the sampling devices and analytical techniques that are used. For example, impaction samplers (e.g. Andersen six-stage impactor, Oxoid air

sampler, and MD8) collect microorganisms on agar plates or gelatine filters, which are extracted or cultured immediately after sampling. The microbial count obtained via this technique is defined as the number of visible colonies on culturable microorganisms' plates (Zhao et al. 2014b). Other techniques (e.g. all-glass impinger, OMNI-3000) collect microorganisms in liquid media that are decimally diluted and then spread on agar plates for culturing following by counting the visible bacterial colonies (Zhao et al. 2014b).

This research project aimed to investigate the total number of airborne bacteria inside of a barn, detect the airborne viruses BoHV-1, PI3 and BRSV, and then establish correlations with environmental conditions including temperature and relative humidity, which is related to the incidence of BRD. In the first part of this study, we determined the optimal air volume and sampling time for both an Oxoid sampler and an MD8 air sampler for collecting total bacteria and viruses, respectively. The results obtained with these volumes (10 L of air on BA plates and 25 L of air on EMB plates using an Oxoid air sampler) were relatively consistent with low variance. Moreover, the MD8 sampler chose 800 L, which required a six-minute sampling time. However, it might be that these air volumes only apply to this particular time (May to July) on the particular farm used in the study and might not apply to other farm studies because of the variation between air samplers. Moreover, they might be dependent on many factors inside of the barn, such as the number of calves, type of ventilation, temperature, humidity, and health status of the calves (Zhao et al. 2014b; Verreault et al. 2008). There are limitations to this study, mainly with regard to the sample size and the number of farms. Previous studies have used several air sampler devices with different air volumes and shown various microbial counts and viral presence, and it remains unclear as to which air

samplers best detect airborne microorganisms. For instance, Islam et al. (2019) collected air samples from a calf barn for 20 min using a liquid cyclone air sampler. They found different airborne bacterial counts inside of the barn, and the number of microbes was impacted significantly by outdoor (rather than indoor) temperature. Moreover, the cleanliness of the barn and the health status of calves play an important role in microbial counts (Lago et al. 2006).

Differences in the number of airborne bacteria inside of the barn might be related to the types and efficiency of air sampler devices, the air volume, and the sampling duration. Further research is needed to test the efficiency of air samplers in livestock houses and develop new air samplers for detecting microorganisms in low concentrations in the air that might spread diseases. These samplers should have high physical and biological efficiency and also accelerate the flow rates to minimise the disruption of calves. For example, Zhao et al. (2014b) tested the physical and biological efficiency and the detection limits of four samplers (Andersen six-stage impactor, all-glass impinger "AGI-30", OMNI-3000, and MD8 with gelatine filter) in collecting aerosols of infectious bursal disease virus. They found that MD8 was generally considered to be more effective than the other devices, with its efficiency potentially reaching 100% for the detection of RNA viruses. A limitation within this experimental study was that the study was conducted to detect only one RNA virus with no changes in environmental conditions such as temperature and humidity. Because RNA viruses are very sensitive to environmental conditions, it would be better to test the air samplers in different environmental conditions to establish correlations with seasonal variation.

Airborne pathogens are generally produced by cattle's skin, faeces and bedding, but pathogens can also be exhaled and coughed into the air by cattle with BRD

(Cambra-López et al. 2010). This study shows different bacterial counts on BA and EMB plates, and the bacteria were different from site to site. What is more, the number of gram-positive bacteria on BA plates was higher than that of gram-negative bacteria on EMB plates during all weeks of sampling. Furthermore, there was no correlation between the temperature and humidity inside of the barn and the airborne bacterial counts on BA and EMB plates. Thus, outdoor environmental temperature may play an important role in bacterial counts inside of a barn (rather than indoor temperature) (Islam et al. 2019), which was not measured in this current study. The bacterial counts in poorly ventilated calf barns associated with BRD frequently approach 100,000 CFU/m^3, and *Staphylococci*, *Streptococci*, *Bacillus*, and *E. coli* usually dominate bacterial plates (Nordlund 2008). Since the present study did not identify the types of microorganisms present in the air of the calf barn, but rather measured only the bacterial counts, we can only compare the total bacterial counts in different sites inside of the barn and establish correlations with the environmental conditions inside of the barn.

Regardless of the housing style, Roque et al. (2016) found that the majority of microorganisms found in the indoor air of swine, chicken and cattle farms were gram-positive bacteria, which are considered to be non-pathogenic (Wathes et al. 1984). According to the results of various research work, the average number of airborne bacteria on BA plates in calf houses was 31,806 CFU/m^3 (Karwowska 2005). Another field study conducted by Lago et al. (2006) found that the average airborne bacteria on BA and EMB plates was 112,280 and 44,482 CFU/m^3, respectively. Moreover, Matković et al. (2007) found that the total number of gram-positive bacteria on a Columbia agar plate inside of a calf barn was 114,000 CFU/m^3. The variation between the studies' results could depend on the number of

barns included, the number of calves, the seasonal variation, and the health status of calves (Quintana et al. 2020). Most studies in the field of airborne diseases have focused only on the numbers of bacterial pathogens present in calf barns. It is possible that the gram-positive bacteria may be non-pathogenic inside of the barn, but could play an important role in increasing gram-negative bacteria and fungi, or might expose the calves to stress, leaving them more susceptible to BRD. Therefore, improving air hygiene can reduce the microbial counts inside of a barn. In most cases, using positive-pressure ventilation systems that deliver small amounts of air to each pen can help to improve the air quality and reduce the microbial counts inside of a barn. Applying these recommendations can produce calf barns that seem to equal calf hutches in minimising disease (Nordlund 2008; Roque et al. 2016). More research is needed to apply standard regulations concerning the permitted number of bacteria and fungi inside of a barn and to better understand this variation between the bacterial counts.

The detection results regarding DNA and RNA viruses via MD8 inside of the barn demonstrated that two RNA viruses, i.e. PI3 and BRSV, were present on the farm, whereas a DNA virus was not detected (BoHV-1). There has been some suggestion that PI3, BRSV, and other viruses in calf barns may be affected by seasons, and the peak of an outbreak occurs between October and March (Pardon et al. 2020). However, this study was conducted between May and July; therefore, it could be that viruses such as BoHV-1 were absent or present in low quantities due to the variation in temperature. Anderson et al. (2016) used bioaerosol, oral fluid, and environmental swab samples in China over several months. They discovered that the temperature inside of a swine barn was a significant indicator of influenza A virus positivity in bioaerosol samples in the autumn and winter months. Moreover,

differences in the design of air samplers may have an impact on these findings. However, it could be that BPI3, BRSV and BoHV-1 were present in relatively low quantities inside of the barn during all weeks of sampling and would be detected with a longer sampling time (collecting more litres of air). What is more, MD8 uses dry capture, which may lead to underestimating the airborne virus concentration or damaging the virus structure during sample collection or transportation. However, the detection of viruses in liquid media is less damaging to viruses than is dry capture, which could result in structural damage and viral desiccation. Damage to a virus structure will result in decreased detection or no detection at all in qPCR as well as an underestimation of the virus concentration (Zhao et al. 2014b).

Having demonstrated that air sampling within a calf barn via an MD8 sampler is able to detect viral RNA and bacterial DNA, it would be possible to extend this study in order to understand more about the bacteria and viruses contained within the air environment by performing deep sequencing of sampled RNA and DNA.

In this study, the sampled farm carried out vaccination schemes against PI3 and BRSV, but not against BoHV-1. Therefore, due to the live attenuated vaccine, it is possible that the viruses were detected in the barn because the calves shed the vaccine strain and spread it within the barn. It would have been better had we used viral sequencing to differentiate between the field and vaccine strains. There are no previous studies on the distribution of PI3 and BRSV in the air inside of barns. However, a seroprevalence study conducted on 756 calves with no vaccination programme against PI3 and BRSV found that 90% of the calves were positive for BRSV and 88% for PI3 (Solís-Calderón et al. 2007). Moreover, Thonur et al. (2012) conducted a study to detect BoHV-1, PI3 and BRSV in 541 calves using swabs and bronchial alveolar lavage samples. They found that 8% of the sample were positive

for BoHV-1, and 2.5% and 5% of the sample were positive for PI3 and BRSV, respectively. Therefore, supporting our findings, upper respiratory tract swabs for pre-weaning calves would be beneficial in determining and confirming the bacteria and viruses associated with BRD via qPCR. The current findings will contribute to the development of improved methods for collecting airborne bacterial and viral pathogens in the indoor air of calf barns, with the aim of reducing airborne pathogens and the transmission of infectious diseases from calf barns.

References

Aich, P., Babiuk, L.A., Potter, A.A., and Griebel, P. (2009) 'Biomarkers for Prediction of Bovine Respiratory Disease Outcome'. *OMICS A Journal of Integrative Biology,* 13, 199-209.

AlHammadi, M. (2016) 'Serological Surveillance of Infectious Bovine Rhinotracheitis Virus, Bovine Viral Diarrhea Virus and Bovine Parainfluenza-3 Virus in Saudi Arabia'. *Alexandria Journal of Veterinary Science*s, 21, 51-48.

Amer, H.M., Abd El Wahed, A., Shalaby, M.A., Almajhdi, F.N., Hufert, F.T., and Weidmann, M. (2013) 'A New Approach for Diagnosis of Bovine Coronavirus Using a Reverse Transcription Recombinase Polymerase Amplification Assay'. *Journal of Virological Methods,* 193, 337–340.

Anderson, B.D., Ma, M., Xia, Y., Wang, T., Shu, B., Lednicky, J.A., Ma, M.-J., Lu, J., and Gray, G.C. (2016) 'Bioaerosol Sampling in Modern Agriculture: A Novel Approach for Emerging Pathogen Surveillance?' *Journal of Infectious Diseases,* 214, 537–545.

Bartholomew, J.W. and Mittwer, T., (1952). The gram stain. *Bacteriological reviews,* 16,1-29.

Besser, T.E., Gay, C.C., and Pritchett, L. (1991) 'Comparison of Three Methods of Feeding Colostrum to Dairy Calves.' *Journal of the American Veterinary Medical Association,* 198, 419–422.

Blachere, F.M., Lindsley, W.G., Weber, A.M., Beezhold, D.H., Thewlis, R.E., Mead, K.R., and Noti, J.D. (2018) 'Detection of an Avian Lineage Influenza A(H7N2) Virus in Air and Surface Samples at a New York City Feline Quarantine Facility'. *Influenza and Other Respiratory Viruses,* 12, 613–622.

Booker, C.W. and Lubbers, B. V. (2020) 'Bovine Respiratory Disease Treatment Failure'. *Veterinary Clinics of North America: Food Animal Practice,* 36, 487–496.

Boshra, H., Cao, J., and Babiuk, S. (2016) *Generation of Recombinant Capripoxvirus Vectors for Vaccines and Gene Knockout Function Studies,* 10, 151–161.

Brownlie, J., Clarke, M.C., Howard, C.J., and Pocock, D.H. (1987) 'Pathogenesis and Epidemiology of Bovine Virus Diarrhoea Virus Infection of Cattle.' *Annales de Recherches Veterinaires. Annals of Veterinary Research,* 18, 157–66.

Buhman, M. J., L. J. Perino, M. L Galyean, T. E. Wittum, T. H. Montgomery, and R. S. Swingle.2000. Association between changes in eating and drinking behaviors and respiratory tract disease in newly arrived calves at the feedlot. Am. J. Vet. Res. 61:1163-1168.

Callan, R.J. and Garry, F.B. (2002) 'Biosecurity and Bovine Respiratory Disease'. *Veterinary Clinics of North America: Food Animal Practice*, 1, 57–77.

Cambra-López, M., Aarnink, A.J.A., Zhao, Y., Calvet, S., and Torres, A.G. (2010) 'Airborne Particulate Matter from Livestock Production Systems: A Review of an Air Pollution Problem'. in *Environmental Pollution*, 158, 1–17.

Caswell, J.L. (2014) 'Failure of Respiratory Defenses in the Pathogenesis of Bacterial Pneumonia of Cattle'. in *Veterinary Path*ology, 51, 393–409.

Caswell, J.L., Bateman, K.G., Cai, H.Y., and Castillo-Alcala, F. (2010) 'Mycoplasma Bovis in Respiratory Disease of Feedlot Cattle'. in *Veterinary Clinics of North America - Food Animal Practice*, 8, 161-186.

Chase, C.C.L. (2013) 'The Impact of BVDV Infection on Adaptive Immunity'. *Biologicals,* 41, 52-60.

Corbeil, L.B. (1996) 'Histophilus Somni Host–Parasite Relationships'. in *Animal Health Research Reviews,* 8, 151-160.

Corzo, C.A., Culhane, M., Dee, S., Morrison, R.B., and Torremorell, M. (2013) 'Airborne Detection and Quantification of Swine Influenza A Virus in Air Samples Collected Inside, Outside and Downwind from Swine Barns'. *PLoS ONE,* 8, e71444.

Chamorro, M.F. and Palomares, R.A. (2020) ‘Bovine Respiratory Disease Vaccination Against Viral Pathogens’. Veterinary Clinics of North America: Food Animal Practice 36 (2), 461–472.

Curtis, G.C., Argo, C.M., Jones, D., and Grove-White, D.H. (2016) ‘Impact of Feeding and Housing Systems on Disease Incidence in Dairy Calves’. *Veterinary Record,* 179, 512–550.

Dagleish, M.P., Finlayson, J., Bayne, C., MacDonald, S., Sales, J., and Hodgson, J.C. (2010) ‘Characterization and Time Course of Pulmonary Lesions in Calves after Intratracheal Infection with Pasteurella Multocida A:3’. *Journal of Comparative Pathology,* 142, 157–169.

Dane, H., Duffy, C., Guelbenzu, M., Hause, B., Fee, S., Forster, F., McMenamy, M.J., and Lemon, K. (2019) ‘Detection of Influenza D Virus in Bovine Respiratory Disease Samples, UK’. *Transboundary and Emerging Diseases,* 66, 2184–2187.

Devi, S. and Mui, W.S. (2010) ‘Laboratory Diagnosis of Dengue Infection’. in *Dengue Virus: Detection, Diagnosis and Control* 139–161.

Dixit, V.D., Marahrens, M., and Parvizi, N. (2001) ‘Transport Stress Modulates Adrenocorticotropin Secretion from Peripheral Bovine Lymphocytes’. *Journal of Animal Science,* 79, 729–734.

Dong, S. and Yao, M. (2010) ‘Exposure Assessment in Beijing, China: Biological Agents, Ultrafine Particles, and Lead’. *Environmental Monitoring and Assessment,* 170, 331–343.

DuBois, W.R., Cooper, V.L., Duffy, J.C., Dean, D.D., Ball, R.L., and Starr Jr., B.D. (2000) ‘A Preliminary Evaluation of the Effect of Vaccination with Modified Live Bovine Viral Diarrhea Virus (BVDV) on Detection of BVDV Antigen in Skin Biopsies Using Immunohistochemical Methods.’ *Bovine Practitioner,* 34, 98–100.

Duff, G.C. and Galyean, M.L. (2007) ‘Board-Invited Review: Recent Advances in Management of Highly Stressed, Newly Received Feedlot Cattle’. *Journal of Animal Science,* 85, 823–840.

Dungan, R.S., Leytem, A.B., and Bjorneberg, D.L. (2011) 'Concentrations of Airborne Endotoxin and Microorganisms at a 10,000-Cow Open-Freestall Dairy1'. *Journal of Animal Science,* 89, 3300–3309.

El-Deeb, W., Elsohaby, I., Fayez, M., Mkrtchyan, H. V., El-Etriby, D., and ElGioushy, M. (2020) 'Use of Procalcitonin, Neopterin, Haptoglobin, Serum Amyloid A and Proinflammatory Cytokines in Diagnosis and Prognosis of Bovine Respiratory Disease in Feedlot Calves under Field Conditions'. *Acta Tropica,* 204.

Ellis, J.A. (2010) 'Bovine Parainfluenza-3 Virus'. *Veterinary Clinics of North America - Food Animal Practice,* 26, 575–593.

Ellis, J.A. (2009) 'Update on Viral Pathogenesis in BRD.' *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*,10, 149–153.

Farmed Animal Antimicrobial Stewardship Initiative. 2021. What is Causing Bovine Respiratory Disease? - Farmed Animal Antimicrobial Stewardship Initiative. [online] Available at: <https://www.amstewardship.ca/faast-reviews/veal-industry/what-is-causing-bovine-respiratory-disease/> [Accessed 23 September 2021].

Fell, L.R., Colditz, I.G., Walker, K.H., and Watson, D.L. (1999) 'Associations between Temperament, Performance and Immune Function in Cattle Entering a Commercial Feedlot'. *Australian Journal of Experimental Agriculture,* 39,795–802.

Frank, G.H., Briggs, R.E., Duff, G.C., Loan, R.W., and Purdy, C.W. (2002) 'Effects of Vaccination Prior to Transit and Administration of Florfenicol at Time of Arrival in a Feedlot on the Health of Transported Calves and Detection of Mannheimia Haemolytica in Nasal Secretions'. *American Journal of Veterinary Research,* 63, 251–256.

Fulton, R.W. (2009) 'Bovine Respiratory Disease Research (1983–2009)'. *Animal Health Research Reviews,* 10, 131–139.

Fulton, R.W., Ridpath, J.F., Confer, A.W., Saliki, J.T., Burge, L.J., and Payton, M.E. (2003) 'Bovine Viral Diarrhoea Virus Antigenic Diversity: Impact on

Disease and Vaccination Programmes'. in *Biologicals,* 31, 89–95.

Fulton, R. W., & Confer, A. W. (2012). Laboratory test descriptions for bovine respiratory disease diagnosis and their strengths and weaknesses: Gold standards for diagnosis, do they exist? In Canadian Veterinary Journal, 53(7), 754–761.

Grønbæk, L., Westphael, N., Martin, H.L., (2016). Obduktioner kaster lys over årsager til kalvedødelighed. Dansk Veterinærtidsskrifts, 1, 30-33.

Gershwin, L.J., Van Eenennaam, A.L., Anderson, M.L., McEligot, H.A., Shao, M.X., Toaff-Rosenstein, R., Taylor, J.F., Neibergs, H.L., Womack, J., Cohen, N., Dabney, A., Dindot, S., Falconer, L., Seabury, C., Skow, L., Enns, M., Thomas, M., Hagevoort, R., Ross, T., Aly, S., Lehenbauer, T., Tucker, C., Zanella, A., Van Tassell, C., and Neibergs, S. (2015) 'Single Pathogen Challenge with Agents of the Bovine Respiratory Disease Complex'. *PLoS ONE*, 10, 1–23.

Gershwin, L.J., Gunther, R.A., Anderson, M.L., Woolums, A.R., McArthur-Vaughan, K., Randel, K.E., Boyle, G.A., Friebertshauser, K.E., and McInturff, P.S. (2000) 'Bovine Respiratory Syncytial Virus-Specific IgE Is Associated with Interleukin-2 and -4, and Interferon-γ Expression in Pulmonary Lymph of Experimentally Infected Calves'. *American Journal of Veterinary Resear*ch, 61, 291–298.

Gorden, P.J., Plummer, P., (2010). Control, management, and prevention of bovine respiratory disease in dairy calves and cows. *Veterinary Clinics of North America - Food Animal Practice,* 26, 243-259.

Girlando, E.M., 2014. Sampling for airborne influenza virus using RNA preservation buffer: a new approach. The University of Iowa.

Griffin, D. (1997) 'Economic Impact Associated with Respiratory Disease in Beef Cattle.' *The Veterinary Clinics of North America. Food Animal Practice,*13, 367–377.

Grissett, G.P., White, B.J., and Larson, R.L. (2015) 'Structured Literature Review

of Responses of Cattle to Viral and Bacterial Pathogens Causing Bovine Respiratory Disease Complex'. *Journal of Veterinary Internal Medicine,* 29, 770–780.

Guterbock, W.M. (2014) 'The Impact of BRD: The Current Dairy Experience'. Animal Health Research Reviews15 (2), 130–134.

Grooms, D.L. and Keilen, E.D. (2002) 'Screening of Neonatal Calves for Persistent Infection with Bovine Viral Diarrhea Virus by Immunohistochemistry on Skin Biopsy Samples'. *Clinical and Diagnostic Laboratory Immunology,* 9, 898–900.

Hay, K.E., Barnes, T.S., Morton, J.M., Clements, A.C.A., and Mahony, T.J. (2014) 'Risk Factors for Bovine Respiratory Disease in Australian Feedlot Cattle: Use of a Causal Diagram-Informed Approach to Estimate Effects of Animal Mixing and Movements before Feedlot Entry'. *Preventive Veterinary Medicine,*117, 160–169.

Hay, K.E., Morton, J.M., Mahony, T.J., Clements, A.C.A., and Barnes, T.S. (2016) 'Associations between Animal Characteristic and Environmental Risk Factors and Bovine Respiratory Disease in Australian Feedlot Cattle'. *Preventive Veterinary M*edicine, 125, 66-74.

Hodgson, P.D., Aich, P., Manuja, A., Hokamp, K., Roche, F.M., Brinkman, F.S.L., Potter, A., Babiuk, L.A., and Griebel, P.J. (2005) 'Effect of Stress on Viral-Bacterial Synergy in Bovine Respiratory Disease: Novel Mechanisms to Regulate Inflammation'. in *Comparative and Functional Genomics,* 6, 244−250.

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. (1991) 'Detection of Specific Polymerase Chain Reaction Product by Utilizing the 5′ → 3′ Exonuclease Activity of Thermus Aquaticus DNA Polymerase'. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 7276–7280.

Horwood, P.F. and Mahony, T.J. (2011) 'Multiplex Real-Time RT-PCR Detection of Three Viruses Associated with the Bovine Respiratory Disease Complex'.

Journal of Virological Methods, 171, 360–363.

Houe, H. (2003) 'Erratum: Epidemiological Features and Economical Importance of Bovine Virus Diarrhoea Virus (BVDV) Infections.*Veterinary Microbiology*, 93, 275–276.

Islam, M., Ikeguchi, A. and Naide, T., (2019). Concentrations of aerosol numbers and airborne bacteria, and temperature and relative humidity, and their interrelationships in a tie-stall dairy barn. *Animals,* 9,1023.

Jensen, P. A., Schafer, M. P., & CIH, P. (1998). NIOSH manual of analytical methods J. Sampling and Characterization Of Bioaerosols. Retrieved from https://www.cdc.gov/niosh/docs/2003-154/pdfs/chapter-j.pdf

Johnson, F.B. (1990) *Transport of Viral Specimens*. *Clinical Microbiology Reviews, 3, 120- 131.*

Johnson, K.K. and Pendell, D.L. (2017) 'Market Impacts of Reducing the Prevalence of Bovine Respiratory Disease in United States Beef Cattle Feedlots'. *Frontiers in Veterinary Science*, 4, 189.

Jones, C. and Chowdhury, S. (2010a) 'Bovine Herpesvirus Type 1 (BHV-1) Is an Important Cofactor in the Bovine Respiratory Disease Complex'. *Veterinary Clinics of North America - Food Animal Practice,* 26, 303–321.

Jones, M.L. and Allison, R.W. (2007) 'Evaluation of the Ruminant Complete Blood Cell Count'. in *Veterinary Clinics of North America - Food Animal Practice*, *23*,377-402.

Karwowska, E. (2005) 'Microbiological Air Contamination in Farming Environment'. *Polish Journal of Environmental Studies,* 14, 445–449.

Kasimanickam, R. (2010) 'Bovine Respiratory Disease "Shipping Fever" in Cattle'. *Pullman, WA: Veterinary Medicine Extension, Washington State University,* 1–3.

Kim, S.H., Chang, S.Y., Sung, M., Park, J.H., Kim, H. Bin, Lee, H., Choi, J.P., Choi, W.S., and Min, J.Y. (2016) 'Extensive Viable Middle East Respiratory Syndrome (MERS) Coronavirus Contamination in Air and Surrounding Environment in MERS Isolation Wards'. *Clinical Infectious Diseases,* 63,

363–369.

Klima, C.L., Zaheer, R., Cook, S.R., Booker, C.W., Hendrick, S., Alexander, T.W., and McAllister, T.A. (2014) 'Pathogens of Bovine Respiratory Disease in North American Feedlots Conferring Multidrug Resistance via Integrative Conjugative Elements'. *Journal of Clinical Microbiology,* 52, 438–448.

Kingsbury DW (1990). Paramyxoviridae and their replication. In: Fields BN, Knipe DM et al., editors. Fields Virology, 2nd ed. Raven Press: *New York*,945–962.

Lago, A., McGuirk, S.M., Bennett, T.B., Cook, N.B., and Nordlund, K.V. (2006) 'Calf Respiratory Disease and Pen Microenvironments in Naturally Ventilated Calf Barns in Winter'. *Journal of Dairy Science,* 89, 4014–4025.

Lange, J.L., Thorne, P.S., and Kullman, G.J. (1997) 'Determinants of Culturable Bioaerosol Concentrations in Dairy Barns'. *Annals of Agriculture and Environmental Medicine*,4, 187–194.

Larsen, L.E., Tegtmeier, C., and Pedersen, E. (2001) 'Bovine Respiratory Syncytial Virus (BRSV) Pneumonia in Beef Calf Herds Despite Vaccination'. *Acta Veterinaria Scandinavica*, 42, 113-121.

Lillie, L.E. (1974) 'The Bovine Respiratory Disease Complex.' in *Canadian Veterinary Journal*. Can Vet J, 15, 233.

Lima, S.F., Teixeira, A.G. V., Higgins, C.H., Lima, F.S., and Bicalho, R.C. (2016) 'The Upper Respiratory Tract Microbiome and Its Potential Role in Bovine Respiratory Disease and Otitis Media'. *Scientific Reports,* 6, 1-12.

Liu, Yuan, Ning, Z., Chen, Y., Guo, M., Liu, Yingle, Gali, N.K., Sun, L., Duan, Y., Cai, J., Westerdahl, D., Liu, X., Xu, K., Ho, K. fai, Kan, H., Fu, Q., and Lan, K. (2020) 'Aerodynamic Analysis of SARS-CoV-2 in Two Wuhan Hospitals'. *Nature,* 582, 557–560.

Livak, K.J., Flood, S.J.A., Marmaro, J., Giusti, W., and Deetz, K. (1995) 'Oligonucleotides with Fluorescent Dyes at Opposite Ends Provide a Quenched Probe System Useful for Detecting PCR Product and Nucleic Acid Hybridization'. *Genome Research,* 4, 357–362.

López-Lorenzo, G., López-Novo, C., Prieto, A., Díaz, J.M., Gullón, J., Arnal, J.L., Benito, A., Díaz, P., Panadero, R., Díez-Baños, P., Dalton, K.P., Parra, F., and Fernández, G. (2021) 'Molecular Detection of Myxoma Virus in the Environment of Vaccinated Rabbitries'. *Transboundary and Emerging Diseases,* 68, 1424–1431.

Lorenz, I., Earley, B., Gilmore, J., Hogan, I., Kennedy, E., and More, S.J. (2011) 'Calf Health from Birth to Weaning. III. Housing and Management of Calf Pneumonia'. in *Irish Veterinary Journa*l, 64-14.

Midwest Plan Service, (1990), Mechanical Ventilating Systems for Livestock Housing, *Iowa State University*, Ames, 6.

Manzoor, A., Maqbool, I., Ganaie, Z., Afzal, I., Khan, H., and Zaffe, B. (2019) 'Mitigating Winter Vagaries in Dairy Animals: A Review. *International Journal of Veterinary Sciences and Animal Husbandy*, 4, 01–05.

Martin, S.W., Darlington, G., Bateman, K., and Holt, J. (1988) 'Undifferentiated Bovine Respiratory Disease (Shipping Fever): Is It Communicable?' *Preventive Veterinary Medicin*e, 6, 27–35.

Matković, K., Vučemilo, M., Vinković, B., Šeol, B., Pavičić, Ž., and Matković, S. (2007) Qualitative structure of airborne bacteria and fungi in dairy barn and nearby environment. *ZV 08_07 3 Matkovic Tisk*, 8, 249–253.

McWilliam, J.M. (1959) 'Antibodies to Asian Influenza and Influenza B: Edinburgh, 1958'. British Medical Journal, 1, 473–476.

Muggli-Cockett, N.E., Cundiff, L. V., and Gregory, K.E. (1992) 'Genetic Analysis of Bovine Respiratory Disease in Beef Calves during the First Year of Life.' *Journal of Animal Scienc*e, 70, 2013–2019.

Murray, G.M., More, S.J., Sammin, D., Casey, M.J., McElroy, M.C., O'Neill, R.G., Byrne, W.J., Earley, B., Clegg, T.A., Ball, H., Bell, C.J., and Cassidy, J.P. (2017) 'Pathogens, Patterns of Pneumonia, and Epidemiologic Risk Factors Associated with Respiratory Disease in Recently Weaned Cattle in Ireland'. *Journal of Veterinary Diagnostic Investigation,* 29, 20–34.

Murray, G.M., O'Neill, R.G., More, S.J., McElroy, M.C., Earley, B., and Cassidy,

J.P. (2016) ‘Evolving Views on Bovine Respiratory Disease: An Appraisal of Selected Key Pathogens – Part 1’. in *Veterinary Journal*, 217, 95–102.

Narita, M., Kawashima, K., Kimura, K., Mikami, O., Shibahara, T., Yamada, S., and Sakoda, Y. (2000) ‘Comparative Immunohistopathology in Pigs Infected with Highly Virulent or Less Virulent Strains of Hog Cholera Virus’. in *Veterinary Pathology, 37*, 402-408.

Ng, T.F.F., Kondov, N.O., Deng, X., Van Eenennaam, A., Neibergs, H.L., and Delwart, E. (2015) ‘A Metagenomics and Case-Control Study To Identify Viruses Associated with Bovine Respiratory Disease’. *Journal of Virolo*gy, 89, 5340–5349.

Nicholas, R.A.J. (2011) ‘Bovine Mycoplasmosis: Silent and Deadly’. in *Veterinary Record*, 168, 459–462.

Nicholas, R.A.J. and Ayling, R.D. (2003) ‘Mycoplasma Bovis: Disease, Diagnosis, and Control’. in *Research in Veterinary Science,* 74, 105–12.

Nadis.org.uk. 2020. *NADIS Animal Health Skills - Respiratory Disease In Dairy And Beef Rearer Units*. [online] Available at: <https://nadis.org.uk/disease-a-z/cattle/calf-management/respiratory-disease-in-dairy-and-beef-rearer-units/> [Accessed 29 October 2020].

Noffsinger, T. and Locatelli, L. (2004) ‘Low-Stress Cattle Handling: An Overlooked Dimension of Management.’ *Proc. Meet. Academy of Veterinary Consultants,* 2, 65–78.

Nordlund, K. V. (2008) ‘Practical Considerations for Ventilating Calf Barns in Winter’. *Veterinary Clinics of North America: Food Animal Practice,* 24, 41–54.

Nordlund, K. V. and Halbach, C.E. (2019) ‘Calf Barn Design to Optimize Health and Ease of Management’. in *Veterinary Clinics of North America - Food Animal Practice*, 35, 29–45.

OLAFSON, P., MacCALLUM, A.D., and FOX, F.H. (1946) ‘An Apparently New Transmissible Disease of Cattle’. *The Cornell Veterinarian,* 36, 205-213.

Paccaud, M.F. and Jacquier, C. (1970) ‘A Respiratory Syncytial Virus of Bovine Origin’. *Archiv Für Die Gesamte Virus*forschung, 30, 327–342.

Panciera, R.J. and Confer, A.W. (2010) ‘Pathogenesis and Pathology of Bovine Pneumonia’. *Veterinary Clinics of North America: Food Animal Practice,* 26, 191–214.

Pansri, P., Katholm, J., Krogh, K.M., Aagaard, A.K., Schmidt, L.M.B., Kudirkiene, E., Larsen, L.E., and Olsen, J.E. (2020) ‘Evaluation of Novel Multiplex QPCR Assays for Diagnosis of Pathogens Associated with the Bovine Respiratory Disease Complex’. *Veterinary Journal,* 256, 105-425.

Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., and Ribbens, S. (2020) ‘Pathogen-Specific Risk Factors in Acute Outbreaks of Respiratory Disease in Calves’. *Journal of Dairy Science,*103, 2556–2566.

Patel, N., Patel, R., Rajoriya, J., Ahirwaar, M., Hari, R., and Ojha, B. (2017) ‘Bovine Respiratory Disease Complex: A Critical Review’. *International Journal of Livestock Research,* 7,23-34.

Paton, D.J. and Taylor, G. (2011) ‘Developing Vaccines against Foot-and-Mouth Disease and Some Other Exotic Viral Diseases of Livestock’. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366, 2774–2781.

Perino, L.J. and Apley, M.D. (1998) ‘Clinical Trial Design in Feedlots.’ *The Veterinary Clinics of North America. Food Animal Practice,* 14, 343–365.

Phillippo, M., Arthur, J.R., Price, J., and Halliday, G.J. (1987) ‘The Effects of Selenium, Housing and Management on the Incidence of Pneumonia in Housed Calves’. *Veterinary Record*, 121, 509–512.

Popescu, S., Borda, C., and Diugan, E.A. (2011) ‘Microbiological Air Quality in Tie-Stall Dairy Barns and Some Factors That Influence It’. *African Journal of Agricultural Research,* 6, 6726–6734.

Poulsen, K.P. and McGuirk, S.M. (2009) ‘Respiratory Disease of the Bovine Neonate’. *Veterinary Clinics of North America: Food Animal Practice,* 25, 121–137.

Prieto, A., Díaz-Cao, J.M., Fernández-Antonio, R., Panadero, R., Díaz, P., López,

C., Morrondo, P., Díez-Baños, P., and Fernández, G. (2014) ‘Application of Real-Time PCR to Detect Aleutian Mink Disease Virus on Environmental Farm Sources’. *Veterinary Microbiology,* 173, 355–359.

Prieto, A., Fernández-Antonio, R., Díaz-Cao, J.M., López, G., Díaz, P., Alonso, J.M., Morrondo, P., and Fernández, G. (2017) ‘Distribution of Aleutian Mink Disease Virus Contamination in the Environment of Infected Mink Farms’. *Veterinary Microbiology,* 204, 59–63.

Pritchard, D.G. (1982) ‘Social and Management Factors Involved in Respiratory Disease of Calves’. *Applied Animal Ethology*, 9, 198-199.

Prost, K., Kloeze, H., Mukhi, S., Bozek, K., Poljak, Z., and Mubareka, S. (2019) ‘Bioaerosol and Surface Sampling for the Surveillance of Influenza A Virus in Swine’. *Transboundary and Emerging Diseases,* 66, 1210–1217.

Philippe Le Mercier, P., 2022. Respirovirus ~ ViralZone. [online] Viralzone.expasy.org.

Available at: <https://viralzone.expasy.org/87?outline=all_by_species> [Accessed 13 January 2022].

Quintana, Á.R., Seseña, S., Garzón, A., and Arias, R. (2020) ‘Factors Affecting Levels of Airborne Bacteria in Dairy Farms: A Review’. *Animals,* 10, 526.

Rahman, M.T., Uddin, M.S., Sultana, R., Moue, A., and Setu, M. (2013) ‘Polymerase Chain Reaction (PCR): A Short Review’. *Anwer Khan Modern Medical College Journal,* 4, 30–36.

Rahpaya, S.S., Tsuchiaka, S., Kishimoto, M., Oba, M., Katayama, Y., Nunomura, Y., Kokawa, S., Kimura, T., Kobayashi, A., Kirino, Y., Okabayashi, T., Nonaka, N., Mekata, H., Aoki, H., Shiokawa, M., Umetsu, M., Morita, T., Hasebe, A., Otsu, K., Asai, T., Yamaguchi, T., Makino, S., Murata, Y., Abi, A.J., Omatsu, T., and Mizutani, T. (2018) ‘Dembo Polymerase Chain Reaction Technique for Detection of Bovine Abortion, Diarrhea, and Respiratory Disease Complex Infectious Agents in Potential Vectors and Reservoirs’. *Journal of Veterinary Science,* 19, 350–357.

Rahmani, A.R., Leili, M., Azarian, G. and Poormohammadi, A., (2020). Sampling and detection of corona viruses in air: A mini review. *Science of The Total Environment,* 740, 140-207.

Rhodes, V., Ryan, E.G., Hayes, C.J., McAloon, C., O'Grady, L., Hoey, S., Mee, J.F., Pardon, B., Earley, B., and McAloon, C.G. (2021) 'Diagnosis of Respiratory Disease in Preweaned Dairy Calves Using Sequential Thoracic Ultrasonography and Clinical Respiratory Scoring: Temporal Transitions and Association with Growth Rates'. *Journal of Dairy Science, 104*,11165-11175.

Ridpath, J.F., Bolin, S.R., and Dubovi, E.J. (1994) 'Segregation of Bovine Viral Diarrhea Virus into Genotypes'. *Virology,* 205, 66-74.

Rice, J.A., Carrasco-Medina, L., Hodgins, D.C., and Shewen, P.E. (2007) 'Mannheimia Haemolytica and Bovine Respiratory Disease'. *Animal Health Research Reviews* 8, 117–128.

Roque, K., Lim, G.D., Jo, J.H., Shin, K.M., Song, E.S., Gautam, R., Kim, C.Y., Lee, K., Shin, S., Yoo, H.S., Heo, Y., and Kim, H.A. (2016) 'Epizootiological Characteristics of Viable Bacteria and Fungi in Indoor Air from Porcine, Chicken, or Bovine Husbandry Confinement Buildings'. *Journal of Veterinary Science*,17, 531–538.

Sachse, M.J.C.I.L.K., Nicholas, L.K.F.R.A.J., and Ayling, R.D. (2018) Gap Analysis of Mycoplasma Bovis Disease , Diagnosis and Control : An Aid to Identify Future Development Requirements, 91–109.

Schaefer, A.L., Cook, N.J., Bench, C., Chabot, J.B., Colyn, J., Liu, T., Okine, E.K., Stewart, M., and Webster, J.R. (2012) 'The Non-Invasive and Automated Detection of Bovine Respiratory Disease Onset in Receiver Calves Using Infrared Thermography'. *Research in Veterinary Scienc*e, 93, 928–935.

Schneider, M.J., Tait, R.G., Busby, W.D., and Reecy, J.M. (2009) 'An Evaluation of Bovine Respiratory Disease Complex in Feedlot Cattle: Impact on Performance and Carcass Traits Using Treatment Records and Lung Lesion Scores.' *Journal of Animal Science,* 87, 1821–1827.

Shephard, R. (2001) 'Viral Diseases of Cattle'. *Australian Veterinary Journ*al, 79, 831–831.

Sherwin, G. and Down, P. (2018) ‘Calf Immunology and the Role of Vaccinations in Dairy Calves’. *In Practice,* 40, 102–114.

Singh, K., Ritchey, J.W., and Confer, A.W. (2011) ‘Mannheimia Haemolytica: Bacterial-Host Interactions in Bovine Pneumonia’. *Veterinary Pathology,* 48, 338–348.

Snowder, G. (2009) ‘Genetics, Environment and Bovine Respiratory Disease.’ in *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases,* 10, 117-119.

Snowder, G.D., Van Vleck, L.D., Cundiff, L. V., and Bennett, G.L. (2006) ‘Bovine Respiratory Disease in Feedlot Cattle: Environmental, Genetic, and Economic Factors.’ *Journal of Animal Science,* 84, 1999–2008.

Solís-Calderón, J.J., Segura-Correa, J.C., Aguilar-Romero, F., and Segura-Correa, V.M. (2007) ‘Detection of Antibodies and Risk Factors for Infection with Bovine Respiratory Syncytial Virus and Parainfluenza Virus-3 in Beef Cattle of Yucatan, Mexico’. *Preventive Veterinary Medicine,* 82, 102–110.

Statham, J.M. (2013) *Investigating Bovine Respiratory Disease and Associated Farm Level Risk Factors : A Pilot Study*, 1–40.

Swida, M.K.- and Stefa, I. (2019) *Pathogenicity and Virulence of Trueperella Pyogenes : A Review*. *Int J Mol Sci, 20*, 27-37.

Sowiak, M., Bródka, K., Buczyńska, A., Cyprowski, M., Kozajda, A., Sobala, W., and Szadkowska-Stańczyk, I. (2012) ‘An Assessment of Potential Exposure to Bioaerosols among Swine Farm Workers with Particular Reference to Airborne Microorganisms in the Respirable Fraction under Various Breeding Conditions’. Aerobiologia 28 , 121–133.

Teagasc.ie. 2022. Teagasc | Agriculture and Food Development Authority. [online] Available at: <https://www.teagasc.ie/> [Accessed 5 January 2022].

Thonur, L., Maley, M., Gilray, J., Crook, T., Laming, E., Turnbull, D., Nath, M., and Willoughby, K. (2012) ‘One-Step Multiplex Real Time RT-PCR for the Detection of Bovine Respiratory Syncytial Virus, Bovine Herpesvirus 1 and Bovine Parainfluenza Virus 3’. *BMC Veterinary Research*, 8, 1–9.

Thomas, A. C., Bailey, M., Lee, M. R. F., Mead, A., Morales-Aza, B., Reynolds, R., Vipond, B., Finn, A., & Eisler, M. C. (2019). Insights into Pasteurellaceae carriage dynamics in the nasal passages of healthy beef calves. Scientific Reports, 9, 1–14.

Timsit, E., Bareille, N., ... Assié, S., 2011. Visually undetected fever episodes in newly received beef bulls at a fattening operation: Occurrence, duration, and impact on performance. *Journal of Animal Science,* 89, 4272–4280.

Urban-Chmiel, R. and Grooms, D. (2012) 'Prevention and Control of Bovine Respiratory Disease'. *Journal of Livestock Science,* 3, 27–36.

Valarcher, J.F. and Taylor, G. (2007) 'Bovine Respiratory Syncytial Virus Infection'. in *Veterinary Rese*arch, 38, 153–180.

Veir, J.K. and Lappin, M.R. (2010) 'Molecular Diagnostic Assays for Infectious Diseases in Cats'. in *Veterinary Clinics of North America - Small Animal Practice, 40*, 1189.

Veit, H.P. and Farrell, R.L. (1978) 'The Anatomy and Physiology of the Bovine Respiratory System Relating to Pulmonary Disease'. in *CORNELL VET,* 68, 555-581.

Verreault, D., Moineau, S., and Duchaine, C. (2008) 'Methods for Sampling of Airborne Viruses.' *Microbiology and Molecular Biology Reviews,* 72, 413–44.

Vissers, M.M.M., Te Giffel, M.C., Driehuis, F., De Jong, P., and Lankveld, J.M.G. (2007) 'Minimizing the Level of Bacillus Cereus Spores in Farm Tank Milk'. *Journal of Dairy Science,* 90, 3286–3293.

Waltner-Toews, D., Martin, S.W., and Meek, A.H. (1986) 'Dairy Calf Management, Morbidity and Mortality in Ontario Holstein Herds. II. Age and Seasonal Patterns'. *Preventive Veterinary Medicine,* 4, 125–135.

Wathes, C.M., Howard, K., Jones, C.D.R., and Webster, A.J.F. (1984) 'The Balance of Airborne Bacteria in Calf Houses'. *Journal of Agricultural Engineering Research,* 30, 81–90.

White, B.J. and Renter, D.G. (2009) 'Bayesian Estimation of the Performance of

Using Clinical Observations and Harvest Lung Lesions for Diagnosing Bovine Respiratory Disease in Post-Weaned Beef Calves'. *Journal of Veterinary Diagnostic Investigation,* 21, 446–453.

Wittum, T.E., Grotelueschen, D.M., Brock, K. V., Kvasnicka, W.G., Floyd, J.G., Kelling, C.L., and Odde, K.G. (2001) 'Persistent Bovine Viral Diarrhoea Virus Infection in US Beef Herds'. *Preventive Veterinary Medicine,* 49, 83–94.

Wolfger, B., Timsit, E., White, B.J., and Orsel, K. (2015) 'A Systematic Review of Bovine Respiratory Disease Diagnosis Focused on Diagnostic Confirmation, Early Detection, and Prediction of Unfavorable Outcomes in Feedlot Cattle'. *Veterinary Clinics of North America - Food Animal Practice*, 31, 351–365.

Wisconsin Uo. Calf Respiratory Scoring Chart [Calf Respiratory Scoring Chart]. [online] Available at: <https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/calf-health-scorer-chs/> [Accessed 20 January 2022].

Zhao, Y., Aarnink, A.J.A., De Jong, M.C.M., and Groot Koerkamp, P.W.G. (2014a) 'Airborne Microorganisms From Livestock Production Systems and Their Relation to Dust'. *Critical Reviews in Environmental Science and Technology,* 44, 1071–1128.

Zhao, Y., Aarnink, A.J.A., Wang, W., Fabri, T., Groot Koerkamp, P.W.G., and de Jong, M.C.M. (2014b) 'Airborne Virus Sampling: Efficiencies of Samplers and Their Detection Limits for Infectious Bursal Disease Virus (IBDV)'. *Annals of Agricultural and Environmental Medicine,* 21, 464–471.

Zucker, B.A., Trojan, S., Müller, W., (2000). Airborne gram-negative bacterial flora in animal houses. *Journal of Veterinary Medicine,* 47, 37–46.