THE IMPACT OF A NEW METHOD FOR THE DETECTION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS ON THE CONTROL OF JOHNE'S DISEASE IN DAIRY CATTLE

Zara Elizabeth Gerrard BSc (Hons) MSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

2017

Supervisors

Dr Catherine Rees

Prof Jonathon Huxley

Dr Ross Davidson

Prof Mike Hutchings

ABSTRACT

Johne's disease (JD) is a severe wasting disease of ruminants, characterised by chronic enteritis, reduction in milk yield, and severe weight loss despite a maintained appetite. The causative agent is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a slow growing pathogen that can take up to 18 weeks for detection on solid culture. Control programmes rely on sensitive diagnostics to identify infected animals quickly so they can be either removed from the herd or managed differently to control the spread of disease. Unfortunately, the Gold Standard of detection is culture, which due to decontamination procedures, has low sensitivity. Enzyme-linked immunosorbent assays (ELISA) are used more often than faecal culture within control programmes as they are cheaper and quicker than culture methods. However, they only detect the animal's immune response, rather than the causative agent. This can cause some issues with diagnosis as the immune response can be affected by other variables. Therefore, to effectively control disease, a new detection method needs to be developed.

In this series of studies, phage-PCR was used within large scale on-farm sampling to establish its performance against the Gold Standard (liquid culture with ESP-trek) and MAP specific antibody milk ELISA (ab-ELISA). Phage-PCR is thought to be more sensitive than other methods due to its low limit of detection. It is also rapid and relatively inexpensive. Results suggest that phage-PCR can detect more animals shedding MAP into their milk than other methods, or in the least a different group of animals than the other methods.

There was some evidence that animals who have had an ab-ELISA positive result in the last year are shedding less MAP into their milk, suggesting that the immune response is helping to control the disease in the short-term. However, this was not observed beyond one year. Phage-PCR had a better agreement with faecal culture than milk culture or ab-ELISA, but this was limited. There was also evidence that early detection could be achieved, as some animals were identified as faecal shedding with phage-PCR before they had seroconversion and detected with ab-ELISA. However, it must be noted that these animals may not be infected and just passaging MAP through the GI tract from the contaminated environment.

An investigation into the prevalence of MAP in pasteurised milk using phage-PCR was also carried out. There is thought to be an association between MAP and Crohn's Disease, with milk highlighted by some as a key transmission vector. There was an increase in the proportion of samples containing viable MAP when compared to other surveys within the literature. However, this was thought to be due to the lower limit of detection that phage-PCR provides, rather than an increase in prevalence.

Phage-PCR can be used effectively for large-scale on-farm sampling to identify animals shedding MAP into the milk. However, some changes to the assay and sample processing will have to be undertaken before this can be used within industry as its current format is laborious and not suited to automation. Until then, it could be used as a tool to further research and understanding into JD in dairy cattle.

ACKNOWLEDGEMENTS

Firstly, my thanks must be extended to my supervisors and laboratory support. Cath Rees for providing microbiology support, Jon Huxley for advice on veterinary matters and putting me in touch with some of my farms, and Mike Hutchings and Ross Davidson for advice on study design, statistics and allowing me to escape to Scotland occasionally! Tania Perehinec, Lorraine Gillett and Ben Swift all provided help in the lab, from mass media runs (sorry Lorraine!), to honing my microbiological skills and teaching me all about mycobacteria.

I could not have got through sampling weeks without my teams of (sometimes) enthusiastic friends. Kate Urwin, Elise Moritz and Megan Godden formed the main on-farm sampling team. Fuelled by cups of tea, Kegworth butcher's sandwiches and McDonald's they braved the 3:30am starts before long days at vet school and brought laughter and entertainment to myself and the herdsmen we were working with. The laboratory team, formed of fellow PhD students, also provided much-needed support and energy which kept me going, especially towards the end of a long day.

The farm owners and herdsmen I have worked with have been a godsend, and I could not have picked a better group if I tried. Thank you for being so generous with your time and allowing me total access to your herds without (too many) questions.

My family also deserve a mention for always believing that I could do this crazy PhD thing, and supporting me all the way.

Finally, I would like to thank my partner Tom Archer, who is hands down the best herdsman I have ever met. He has helped at all stages of this work, from the discussion of future study ideas, to general debates on Johne's disease. He taught me everything I know about dairy cows and the farming industry, suffering through many hours of questioning and being a shoulder to cry on when things were difficult. Without his presence and uplifting energy, most of this would not have been possible, and I owe him everything for that. You deserve your name on this work as much as I do.

Oh.

and I owe it all to the cows.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BHI	Brain-heart infusion
CD	Crohn's disease
CFU	Colony forming unit
CIS	Cattle Information Service
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ab-ELISA	MAP specific antibody ELISA
HEYM	Herrold's egg yolk media
HTST	High-temperature short-time
JD	Johne's disease
LTLT	Low-temperature long-time
M+	Media plus
MAP	*Mycobacterium* avium *subspecies paratuberculosis*
MRD	Maximum recovery diluent
NML	National Milk Laboratories
NMR	National Milk Records
OADC	Oleic acid, bovine albumin fraction, dextrose and catalase
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
REML	Residual maximum likelihood
RO	Reverse osmosis
Se	Sensitivity
Sp	Specificity
SSC	Somatic cell count

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 JOHNE'S DISEASE; A HIDDEN PROBLEM

Johne's disease (JD) is a severe wasting disease of ruminants, classically characterised by chronic enteritis, reduction in milk yield, and severe weight loss despite a maintained appetite. This eventually leads to death (Fecteau and Whitlock, 2010). Other signs can include intramandibular oedema (refered to as "bottle jaw"), and a decrease in fertility, although these signs can be pathognomonic. The disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a slow-growing bacterium, which is usually passed on to young animals via the faecal-oral route during the first few months of life (Sweeney, 1996), with approximately 75 % of calves infected within the first 6 months (Windsor & Whittington, 2010). However, this bacterium can also be passed vertically *in utero* (Seitz *et al*., 1989; Whittington & Windsor, 2009) and also by the ingestion of colostrum from an infected cow (Nielsen *et al*., 2008), making strict management practices on infected farms vital for the control of the disease. It is thought that 69 % of the United Kingdom's dairy herds have been exposed to MAP (NML, unpublished data), making it a disease of high economic impact, with the estimated cost to the UK agricultural industry being upwards of £13 million annually (£9.8 million in dairy and £3.1 million in beef) due to loss in milk yield and increased fertility problems (Caldow & Gunn, 2002). Johne's disease is described as being endemic in the UK and across the globe, with the incidence of the disease rising dramatically since the opening of the Single European Market in 1992 (Mee & Richardson, 2008), which allowed indiscriminate trading between the UK and other EU member states. It is also very likely that

in the UK re-stocking of herds after the 2001 Foot and Mouth Disease outbreak amplified the spread of JD, similarly to that seen in the bovine tuberculosis (bTB) epidemic (Carrique-Mas et al., 2008; Vial et al., 2015).

Johne's disease has a long incubation period, with clinical signs becoming apparent when the cow is two to four years old, often after a period of stress (Mortier *et al*., 2015). By this point, the cow has already been shedding MAP intermittently, possibly passing infection on to its calves and other cows (Marcé *et al*., 2011; van Roermund *et al*., 2007). Due to this problem, early detection is key to disease control. However, current diagnostics are lacking in sensitivity (Se) to do this. New diagnostics and better use of those currently available alongside better designed control programmes will play a key role in controlling this disease.

1.2 AETIOLOGY OF JOHNE'S DISEASE

There are thought to be four stages of MAP infection; silent, subclinical, clinical and advanced (Chacon *et al*., 2004; Tiwari *et al*., 2006). Details of these stages are taken from Fecteau & Whitlock (2010). Silent infection includes cattle of up to two years of age, where there are no clinical signs of infection, no detectable subclinical effects, and no diagnostics are currently sensitive enough to detect low levels of infection. However, these animals could still be shedding MAP intermittently into the environment. Animals with subclinical infection, like silent infection, still do not display any clinical signs, but shedding of MAP may be detected by tests such as faecal culture as numbers of bacteria shed into the gut lumen have generally increased. Cows who start to exhibit gradual weight loss, despite maintaining a normal appetite, loss in milk yield and an increase in scouring fall into the clinical category. At this point, all cows are thought to have intermittent MAP positive faecal cultures, and increased antibody levels detectable by enzyme-linked immunosorbent assay (ELISA). The final stage is advanced clinical. Animals in this category are rarely seen in commercial herds as cattle are normally culled at the clinical stage or possibly before that due to decreased milk production and weight loss (Whitlock & Buergelt, 1996). Merkal *et al*. (1975) found that mastitis and infertility are the main reasons for culling with unapparent paratuberculosis. This stage includes those cows whom have displayed severe weight loss, diarrhoea and intermandibular oedema (Fig. 1.1), alongside other signs including severe lethargy and occasionally anaemia.

Figure 1.1. Clinical signs of JD in cattle

Classic clinical signs of JD include severe weight loss despite maintained appetite and milk loss, which is shown in A compared to healthy cows (Authors own, 2015), and B (Meadows, 2004). Other signs include intramandibular oedema commonly known as bottle jaw, although this is common in other conditions (C; Karreman, n.d.; D; Meadows, 2004)

Traditionally, MAP infection is thought to occur within the first few months of life, when the calves are most susceptible to infection. This is age-dependant, with older calves exhibiting increasing tolerance to MAP, and by one year old calves have a level of resistance that is comparable to that of an adult cow (Sweeney, 2011). Although calves can still become infected at this age, it requires a higher infectious dose and longer exposure to MAP (Fecteau & Whitlock, 2010). A small number of exposed calves are able to clear the infection, with the majority becoming latently infected (Smith *et al*., 2009). Of these latent infections, only 10 % will go on to develop clinical disease (Koets *et al*., 2015). Most of these animals are able to control their latent infection and are never diagnosed as MAP positive.

There are still many unknown aspects of MAP infection, with differences noted between natural infection and experimental infection models complicating the interpretation of data. One example of this is an early oral infection model conducted by Payne & Rankin (1961) using a single high dose of MAP. They suggested that MAP may infect the host as early in the gastrointestinal system as the tonsillar crypts. Further studies using a low dose (Sweeney *et al*., 2006), or trickle dose (characterised as a low dose over numerous weeks; Gilmour *et al*., 1965), suggested that the ilium is the primary site of infection. More recently Sweeney *et al*. (2006) conducted two experiments, the first being a high dose infection of $2.5x10^{10}$ cfu at two and three days old and the second a low dose of either $5x10^{9}$, $5x10^{8}$ or $1.5x10^{6}$ cfu at 21 and 22 days of age. The latter experiment was thought to mimic a natural infection, as it was less over whelming. All calves were found to be

culture positive using tissue samples from the ileum and jejunum. However, those with the low infectious dose were not faecal culture positive at the time of post mortem at 42 days of age. Even though models are not the best tools for infection challenges as it is difficult to mimic natural infection, they are significantly cheaper than natural infection trials where a large number of animals would need to be followed across numerous years to yield statistically robust results.

Genetics also plays a role in susceptibility to infection, and selecting for resistance is emerging as an approach to disease control that can be used alongside other management practices as a preventive measure (Kirkpatrick & Shook 2011). Breeding disease resistance into cattle is a slow long-term process but, unlike other management changes which can slip over time, the results are permanent. Cetinkaya *et al*. (1997) established that there are breed differences, with Channel Island breeds such as Jersey or Guernsey having a higher risk of disease when compared to Friesian (odds ratio >10.9), which suggests that herds who either cross-breed Friesian or Holstein cattle to a Channel Island to improve the butter fat content of the milk, or have a small number of Channel Islands within their main herd, are at higher risk of disease. There are multiple studies that conclude that not only is susceptibility to disease hereditary (Koets *et al*., 2000; Nielsen *et al*., 2002), but also the ability to produce an immune response. For instance Mortensen *et al*. (2004) found that genetics can influence the chance of returning the undesired humoral immune response where the immune system does not control the infection any longer. Koets *et al*. (2010) investigated this further, focusing on

the Toll-like receptor 2 genes (TLR2) which are involved in the recognition of MAP, stimulation of phagocytosis in the macrophage and cell-mediated immunity. They found that MAP-infected cattle were more likely to have a single nucleotide polymorphism (SNP) in the TLR2-1903 region than their uninfected counterparts. This SNP can cause inefficient recognition of MAP by the macrophages, and failure of the subsequent innate and adaptive immune responses, which has been seen in tuberculosis and other mycobacterial infections in humans (Kang & Chae, 2001; Ogus *et al*., 2004). However, this study was only conducted on a small number of cattle on a case-control matched basis (twelve of each). Kirkpatrick & Shook (2011) explain that genetic studies are limited to small numbers of animals or field data as studies of this type are expensive due to the high individual animal cost, slow disease progression and large number of animals needed.

The following sections detail the cow's immune response, or lack thereof, during each stage of infection.

1.2.1 Silent infection

Within a few hours of ingestion MAP can be found within the epithelial macrophages and ileal M cells (Momotani *et al*., 1988; Sigurethardóttir *et al*., 2004); Fig. 1.2). M cells sample the environment within the intestine and present foreign cells to the Peyer's patches where groups of naïve immune cells such as B-cells, T-cells and macrophages are located (Tizard, 2013). It is here, within unactivated macrophages, that MAP finds a perfect environment to replicate and evade the host's immune system (Coussens *et al*., 2010). There are many receptors identified that MAP can utilise to gain access to macrophages, such as the mannose receptor (Astarie-dequeker *et al*. 1999), complement receptor types CR3 and CR4 (Schlesinger & Horwitz, 1991), Toll-like receptor 2 (Means *et al*., 1999) and the fibronectin receptor (Kuroda *et al*., 1993). The fate of the microorganism entering the macrophage depends on which cell-surface receptor that they bind to. For example, by binding to CR3 – which seems to be important in MAP infection – the mycobacteria avoid triggering an oxygen burst (Sigurethardóttir *et al*., 2004). Once inside the phagosome of the macrophage the bacteria appear to interfere with normal phagosome maturation into a phagolysosome, thereby escaping the process of destruction, however the mechanism of this process is unknown (Coussens, 2001). A key factor in the host's defence against bacterial spread during this stage of infection is thought to be the production of interferon gamma (INF-γ) to activate naïve macrophages, but this will do little to cure a persistently infected cell (Coussens, 2001).

1.2.2 Subclinical infection

Subclinical infections are notoriously difficult to diagnose due to low antibody levels, lack of clinical signs, and infrequent or low-level shedding of MAP into the faeces. It is at this stage of infection that granulomas, defined as a localised aggregate of infected and uninfected macrophages (Klinkenberg & Koets, 2015), are formed within the host to contain the infection. However granulomas allow relatively unrestricted growth of MAP inside the infected macrophages where they are shielded from cytotoxic immune effects (Coussens, 2001). Due to the reduction of INF-γ and tumour necrosis factor-alpha (TNF-α) by yet unknown mechanisms, any macrophages recruited to the site of infection will be susceptible to infection by MAP released from granulomas (Khalifeh & Stabel, 2004), therefore facilitating the spread of bacteria.

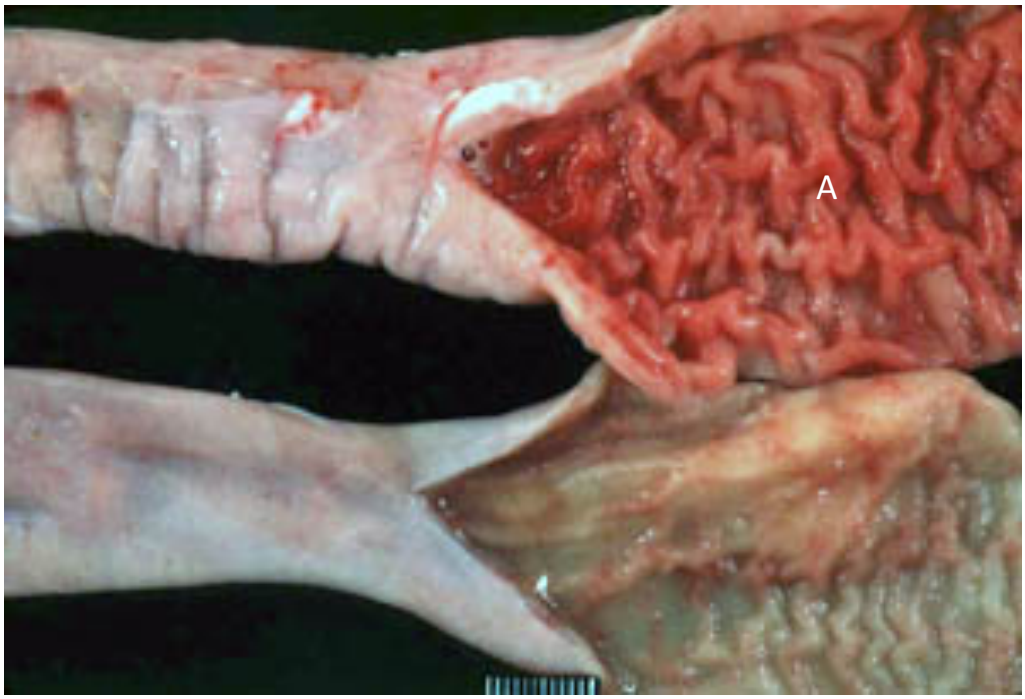
Flesch & Kaufmann (1994) found that mycobacteria can actively induce the expression of Interleukin-10 (IL-10) by macrophage. IL-10, an anti-inflammatory and immunosuppressive cytokine, also plays a role in subclinical infection, as this acts against the host's immune system to supress T-cell activity, and therefore the type-1 immune response, allowing the infection of naïve macrophages (Jacobs *et al*., 2000). This is a normal part of the host's immune response to bacterial pathogens, helping to limit tissue damage caused by the release of proinflammatory cytokines. This suggests that this may be another route used by MAP to evade the host immune response as tissue will become inflamed, producing granulomas as described above.

1.2.3 Clinical and advanced infection

Progression from subclinical to clinical infection is highly variable and influenced by a wide range of factors including but not limited to; age and dose at initial exposure, genetics, environment, nutrition and production stressors (Fecteau & Whitlock, 2010). At the clinical stage of disease, MAP can be found in macrophages within numerous tissues across the body, and also in the blood and mammary glands (Koenig *et al*., 1993). Although, MAP can be found in the blood of even subclinical cows if the detection method is sensitive enough (Swift *et al*., 2013). The release of MAP from infected macrophages results in further damage to the intestinal epithelium causing severe thickening with granulomas and inflammation (Whitlock & Buergelt (1996); Figure 1.2). At this point the type-1 inflammatory and cytotoxic response begins to decrease, leaving a type-2 response only where antibodies are readily produced (Coussens, 2001). However, the antibody response is not able to contain MAP infection and can actually exacerbate the situation since it can promote the uptake of bacteria by the macrophages. Therefore, MAP evades the immune system again by converting a protective inflammatory response into to a humoral response which does little to protect the host.

Due to the thickening of the intestinal wall (Fig. 1.2), the animal is no longer able to absorb nutrients from their food so severe scouring (Fig. 1.3) and emaciation occurs, despite a maintained appetite.

Figure 1.2. Intestine from an infected cow compared to an uninfected cow



Inflammation and thickening of the submucosa caused by infection can be seen (top; A), compared to an intestine from an un-infected cow (Greenstein, 2003).

Figure 1.3. Severe scouring caused by JD

(North Dakota Department of Agriculture, 2017)

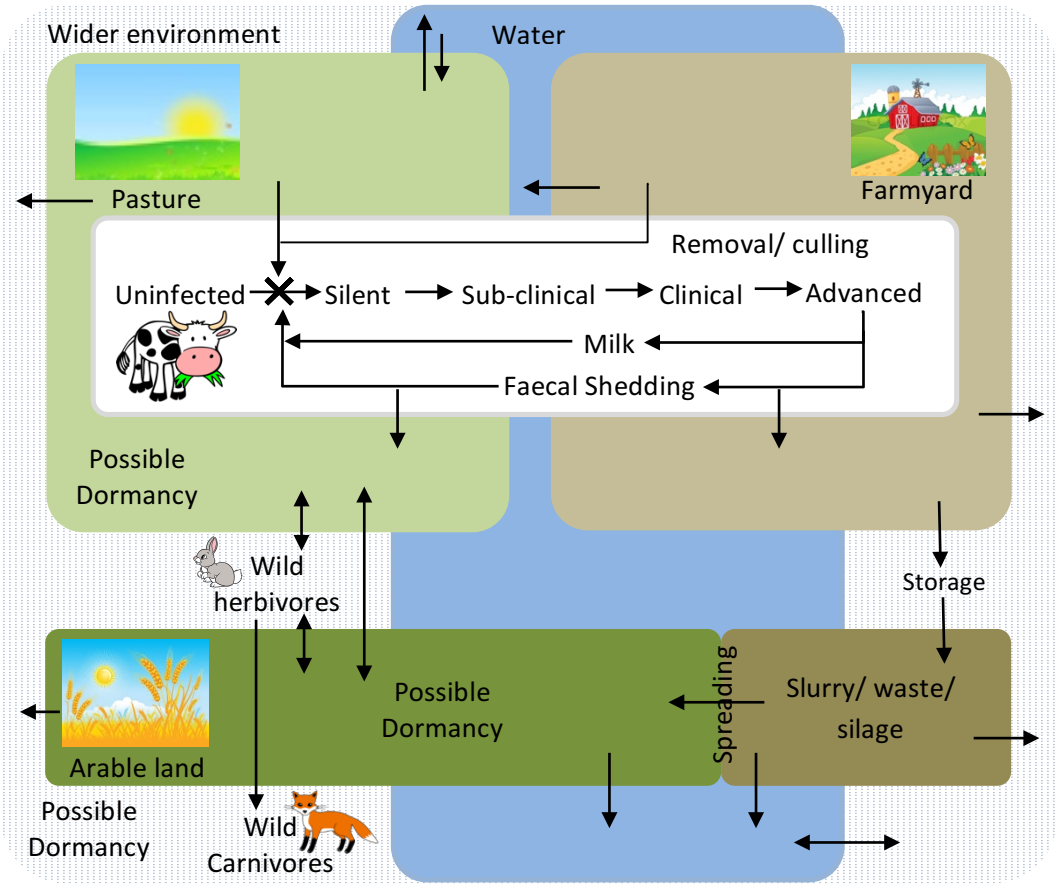
1.3 EPIDEMIOLOGY

Johne's disease is an example of a disease that possesses a high degree of uncertainty and variability in parameter values. In part caused by the low Se of diagnostic tests and the long sub-clinical phase of the disease. However, there are large knowledge gaps that contribute to the uncertainty. This makes it very difficult to estimate prevalence and conduct comprehensive studies (Boelaert *et al*., 2000). Differing environmental and management practises also result in changes in prevalence and transmission rates seen within herds (Davidson *et al*., 2012), adding to the complexity of the problem. Elliott *et al*. (2014) also highlighted the problem of comprehensive studies, explaining that studies of disease management needs to take into account the persistence and transport of bacteria on soil and water, land use, spread via domestic and wild populations, alongside general animal management (Fig. 1.4).

The performance of detection methods also severely affects the ability to diagnose and control JD, with most methods having a low Se, meaning that there is a proportion of cows who will give false negative results. A study by Woodbine *et al*. (2009) within the South West of England highlights the epidemiological complexity of the disease. A total of 29,782 serum samples were collected from 15,736 individual cattle and tested using an indirect ab-ELISA, and it was found that 10.1 % of cattle gave a single positive result. However, when cattle are only considered MAP-positive if they give three consecutive positive test results – a common method adopted widely for JD diagnostics – the positivity rate dropped to 7.1 %. Further to this, Markov

chain Monte Carlo simulation was used by this group to re-estimate the Se and specificity (Sp) of the ab-ELISA, finding that the Se increased from 33.3 – 34.8 % over the three tests and Sp was at 99.7 % for all three. This estimation is markedly better than the original values of 27.8 % for Se and 90.1 % for Sp when faecal culture was used as the Gold Standard to compare the performance of ab-ELISA. The increase of Se and Sp observed when not comparing against an imperfect Gold Standard means that diagnostics may be performing better than expected.

Figure 1.4. Distribution of MAP within the farming environment



Shedding from infected animals can significantly contaminate the surrounding environment. Shedding of MAP from faeces can spread within the pasture and farmyard, into the water courses and wildlife. Environmental MAP can then re-enter the herd during grazing of young stock at pasture. These pathways show that infection does not always come from the dam. (adapted from Elliott *et al*. (2014))

1.3.1 Prevalence

The prevalence of JD in cattle is difficult to estimate. Research efforts are driven by the need to develop effective control and prevention programmes, as too many JD control programmes have been designed on the assumption that disease prevalence is low (Barkema *et al*., 2010). The long and variable incubation period, lasting from a few months to many years, with clinical cases occurring sporadically, means that this disease is a perfect example of the 'iceberg concept'. This is described by Magombedze *et al*. (2013) and explains some of the difficulties in estimating prevalence. Magombedze *et al*. (2013) predict that for every two-to-four cattle diagnosed, another six-to-ten cattle are misdiagnosed actually having sub-clinical or silent disease (Fig. 1.5). This is before considering the variability in the many different diagnostic tests being available, and the probability that only a subset of the infected animals are detected.

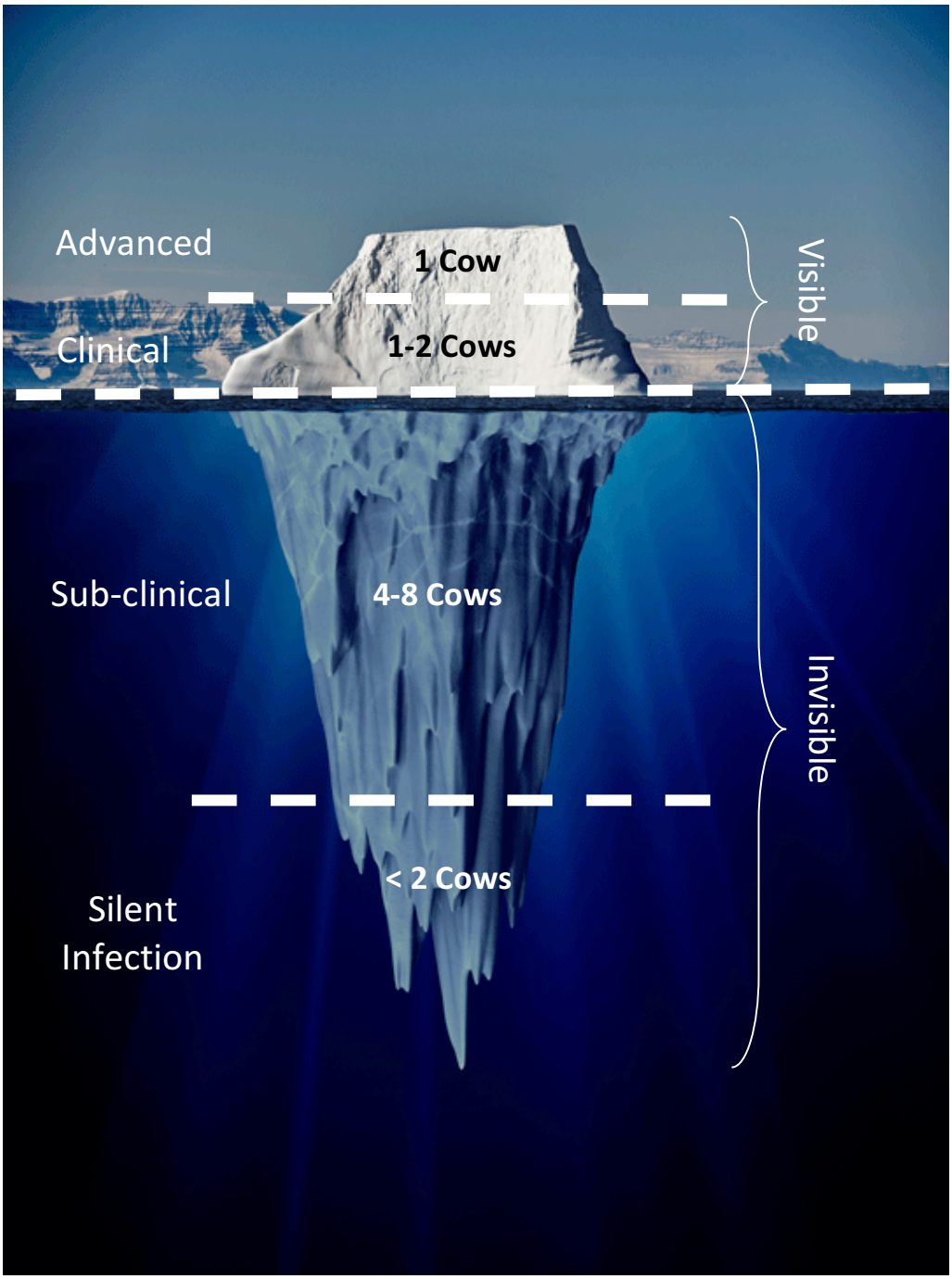
Johne's disease is common in many countries that have significant livestock industry. The estimates for within-herd prevalence, or the number of animals who are MAP positive within a herd, varies dramatically depending on the test used and the region. The Nielsen & Toft (2009) reviewed 30 articles referring to animal-level prevalence between 1990 and 2007 and found that this could range between 0.1 – 24.1 %, primarily due to test differences. Italy and France had the highest herd-level prevalence, with over two-thirds of herds affected by the disease. Conversely, Slovenia and Switzerland had the lowest number of herds affected (less than 4 %). Sweden was apparently JD-free, with 200

herds tested by either faecal or tissue culture, although there is a chance that a small number of infected animals remain due to the Se of the diagnostics used.

According to Nielsen & Toft (2009), the UK had an apparent herd-level prevalence of approximately 17 %, based on observations from farmers located in England and border regions of Wales. This was very similar to the herd-level prevalence reported more recently in Ireland (21 %; Good *et al*. 2009), where herds were classed as positive if at least one cow was ab-ELISA positive. This Irish estimate has been updated further to between 23 and 34 % using a Bayesian estimate (McAloon *et al*., 2016). However, National Milk Records (NMR, n.d.) suggested that 69 % of herds in the UK are infected with JD, an estimation based on farms with at least one positive animal, screened within a six month period in 2011, that had signed up to the Herdwise quarterly milk ab-ELISA testing scheme. Although, this could be the lower end of the estimation due to the propensity for ab-ELISA to miss a number of infected animals.

Good *et al*. (2009) also looked at the animal-level prevalence in Ireland. They conducted a random survey of serum samples collected from the Irish national herd (20,322 cattle within 639 herds) and found that 2.86 % of all animals were MAP positive, increasing to 3.30 % for animals less than two years old, again highlighting the difficulties brought about by testing young animals (Nielsen *et al*., 2002).

Figure 1.5. The iceberg concept to describe JD in a herd



The iceberg concept is used to describe the number of infected cows that go unidentified when estimating the prevalence of JD, as only a group of cows are visible with many more 'under the surface' (adapted from Magombedze *et al*. (2013).

Botsaris *et al*. (2013) conducted a survey in Cyprus of 225 bulk-tank milk samples using phage-PCR (Section 1.6), and found that than 22.2 % (50 of 225 samples) were positive for viable MAP. Bulk-tank samples are not the most accurate way to determine herd prevalence, as there could be an up to 10 % change in within-herd prevalence before the bulk-tank sample will reflect a change due to dilution with milk from non-infected animals. Having acknowledged this limitation, testing bulk-tank milk provides a quick overview of herd disease load, but needs further evaluation as a method to accurately measure herd prevalence (Sweeney *et al*., 2012).

1.3.2 Transmission

1.3.2.1 Vertical transmission

The most important transmission pathway for JD is dam-daughter infection which can occur via multiple routes, most involving oral ingestion of MAP (Fig. 1.6; Sweeney, 1996). The primary route suggested by many authors, is faecal-oral transmission from the environment or contamination of the dam's udder with infected faeces (Fecteau & Whitlock, 2010). However, transmission can also occur via shedding of MAP into the colostrum (Laurin, 2015; Nielsen *et al*., 2008; Stabel *et al*., 2014) and foetal infection *in utero* (Whittington & Windsor, 2009).

Disease control programmes aim to break the faecal-oral transmission route through hygienic calf rearing and removal of infected cows from the herd

(Marcé *et al*., 2011). Benedictus *et al*. (2008) studied a medium-sized dairy farm that had been suffering major losses over a period of around twenty years. The researchers implemented a management-based control programme and aimed to estimate within herd transmission parameters. After the implementation of the control programme, which involved the cleaning and disinfection of the calving pen between calvings, separation of calves from dams as soon as possible, and colostrum only being taken from clean animals then bottle fed to the calves, calves born from infected dams were at a significantly higher risk of becoming MAP infected when compared to calves born from faecal culture negative dams, with 9.5 % and 26.8 % of calves becoming infected respectively. From the start of the programme JD prevalence dropped dramatically from 60 % to less than 20 % providing evidence that a good management programme that reduces transmission can significantly reduce disease load of a herd. This study, although primarily regarding control programmes, provides evidence that dam-daughter transmission, whether by faecal-oral or colostrum, plays an important role in infection of young animals.

Management of cattle during and after birth cannot completely rule out vertical transmission. *In utero* infection by MAP was first reported in 1929, where acid-fast bacilli were visualised and isolated from foetal membranes, blood, liver and other tissues (Whittington and Windsor, 2009). This has been replicated on multiple occasions, including isolation of MAP from ovaries in a small number of clinical cattle in 1958, but doubt was cast on its importance due to lack of methodological descriptions (Whittington and Windsor, 2009).

Whittington & Windsor (2009) conducted a review and meta-analysis of *in utero* infection, finding that around 9 % of foetuses from sub-clinically infected cattle, and 39 % from clinically infected cattle, were infected with MAP, but this was assumed as an underestimate due to the detection methods used. The findings from the meta-analysis provided significant evidence for *in utero* infection, concluding that this method of transmission could limit the success of disease control programmes, which in turn highlights the importance of breeding JD positive animals to a terminal sire. Merkal *et al*. (1982) inoculated MAP into the uterus of thirteen cows 24 h after service to investigate whether the bacteria could survive in that environment. They found that MAP could be isolated from the uterine body and horns, and also the adjacent lymph nodes, indicating potential for survival. Reports of isolation of MAP from foetal tissues extend as far back as 1929, but early reports are sporadic and methods are difficult to replicate (Whittington & Windsor, 2009). The best designed natural infected study by Seitz *et al*. (1989) involved collection of tissue samples from over 400 animals at an abattoir, 392 clinically normal and 15 cows with clinical JD. All foetuses from MAP culture negative animals were also found to be culture negative.

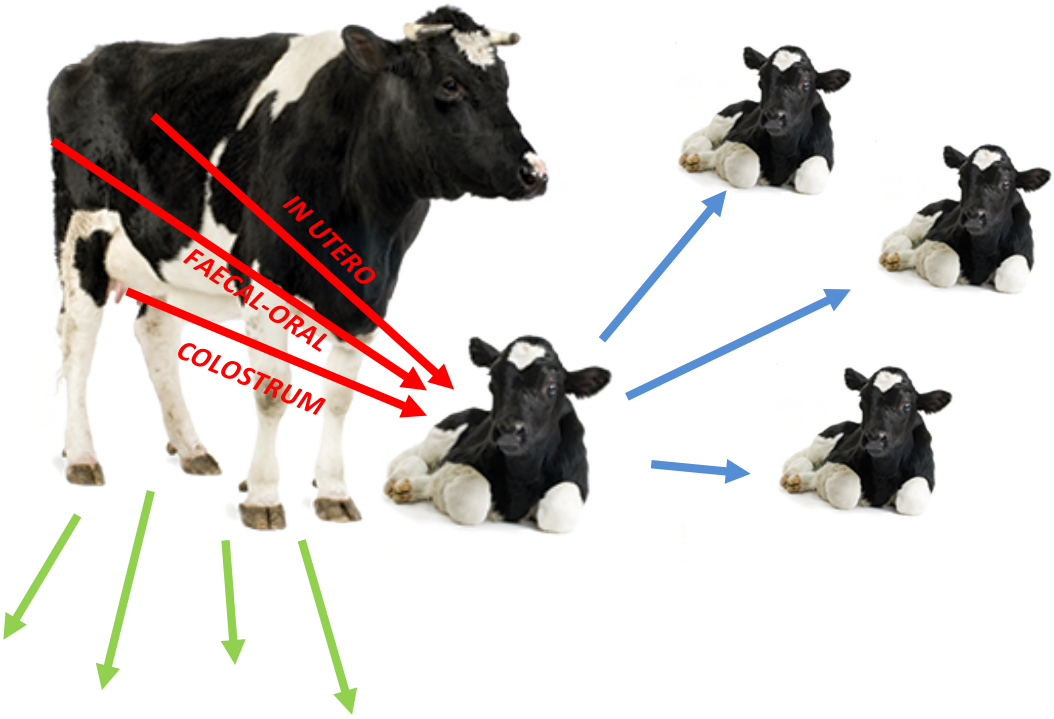
These findings into infection of foetal tissue are important to consider when designing a control programme to reduce cow-calf transmission, as it may seriously hinder its success.

1.3.2.2 Horizontal transmission

Horizontal transmission between adult cows is also possible, albeit posing a much lower risk than dam-daughter. Espejo *et al*. (2013) obtained 79 unexposed and 260 exposed cows and introduced the unexposed cows at various ages into five MAP infected herds. From two years old, faecal and serum samples were collected annually from each animal, and records of culling obtained. When comparing the cohorts, it was found that delayed exposure to MAP resulted in lower incidences of MAP positive culture and ab-ELISA results compared to those exposed since birth, but a risk of infection was still present. They also found that the risk of testing positive for MAP or developing clinical JD increased with time of exposure, regardless of whether they were from the infected or uninfected cohort. This presents a risk for cattle at pasture, or those kept in a contaminated environment. In addition to vertical transmission from the dam, van Roermund *et al*. (2007) found that calf-calf transmission also occurred within an experimentally infected herd. During a three-month observation period the basic reproduction number (R_0) was estimated at 2.7 and 0.9 for cow-calf and calf-calf transmission, respectively. This means that although the risk of transmission remains present, calf-calf transmission would not sustain the epidemic alone as the R_0 is below one, so for every case less than one other animal would become infected . Marcé *et al*. (2011) agreed with van Roermund *et al*. (2007) when they simulated within-herd transmission of MAP within a persistently infected, closed, dairy herd. They found that contacts between calves before weaning did not influence transmission in a herd, whereas the level of

exposure of calves to a contaminated area and the starting age of exposure were more important. This effect was found to be completely independent of herd size. The evidence presented here suggests that transmission and infection of older cows, albeit rare, is something that needs to be considered.

Figure 1.6. Transmission of MAP from an infected dam



Transmission of MAP to young stock within the farm environment can occur via multiple routes. The red arrows represent vertical transmission from dam-to-calf and blue arrows represent horizontal transmission from calf-to-calf, primarily via faecal-oral route. The green arrows represent contamination of the wider calving environment.

1.3.2.3 Survival in the environment

Infected cattle can release as many as 10^8 MAP cells per gram of faeces, and the presence of these animals in the herd increases the likelihood of exposure of others due to how well the bacterium can survive in the environment (Whittington *et al*., 2004). Berghaus *et al*. (2006) conducted environmental testing of lagoon water, manure samples from sick pens and manure from alleyways on 23 large dairy herds in California using culture, then compared these to ab-ELISA and individual and pooled faecal culture results of more than 60 animals in each herd. They found that the estimated prevalence of infected animals did not differ based on the results gained using the different testing methods. They also looked at measures of agreements between the environmental samples and the diagnostic test results, and found that there was a 70 % observed agreement with faecal culture results, compared to 65 % for ab-ELISA results, making environmental sampling a viable alternative to individual animal testing. Worryingly, the use of recycled manure solid (RMS) for bedding has become more popular in recent years due to improvements in technology, enabling drier material to be produced. Leach *et al*. (2015) conducted a review on the benefits and risk of the use of RMS on UK farms, and found that that no studies directly related RMS use to an increase in the incidence or prevalence of infectious diseases other than mastitis. However, considering that MAP can be found in powdered baby milk (Botsaris *et al*., 2016) which has been homogenised, pasteurised and spray dried (FDA, n.d.), it is concerning that this is being considered safe for use.

MAP is able to survive in a variety of conditions, but more so in a dry fully shaded areas where it can survive up to 55 weeks (Whittington *et al*., 2004). Interestingly, the same research group found that MAP can survive on grass germinated through infected faecal material up to 24 weeks. Salgado *et al*. (2011) tested the effects of the application of MAP infected slurry to soils, concluding that although there was a lag time of two months before MAP was detectable in water drainage samples, MAP remained in the top layer of the soil and was detected most often in grass clippings. MAP is widely distributed in soils throughout Britain, with Rhodes *et al*. (2013) finding it in 10.5 % of soil cores collected as part of a nationwide monitoring scheme with a range of 5 x 10^2 to 3 x 10^6 cfu g^{-1} being detected. This of course means that any JD free farm who shares contractors or general machinery with another infected herd are at risk of their livestock picking up MAP from the pasture, increasingly so if the farm is buying in slurry from an infected herd.

Alongside survival in the soil, MAP has the ability to form biofilms. Cook *et al*. (2010) studied the formation of biofilms in water troughs over a 365-day inoculation survey. Concrete, plastic, galvanised and stainless-steel troughs with 32 litres of water collected from a dairy farm located in Kentucky and diluted 1:1 with potable tap water before they were inoculated with a high dose of MAP (1.7 x 10 cfu ml^{-1}). Concentrations of bacteria in biofilms remained the highest in the concrete and galvanised steel troughs, averaging at around 1 x 10^6 cfu cm^{-2}, compared to the plastic and stainless-steel troughs at around 1 x 10^5 cfu cm^{-2}. MAP cells were found to be less than 10 % of the total biofilm population after 21 days, dropping to around 2 % after this. The

organism did not disappear from the biofilms, instead plateauing around 149 days into the experiment. It is not only contamination with faecal matter that could result in the presence of MAP in water troughs. Many authors have found MAP in potable water (Falkinham *et al*., 2001; Mihajlovic *et al*., 2011). However, 42.5 % of UK farmers use water from a bore-hole source (Dairy Co, 2011), meaning that MAP could leach through the soil from slurry applications or grazing stock into the water, as described by Salgado *et al*. (2011).

1.3.2.4 Wildlife reservoirs

There is a risk of interspecies transmission; Stevenson *et al*. (2009) investigated 164 MAP isolates from across seven European countries, collected from nineteen difference species, and found that there is evidence for transmission between wildlife and ruminants on the same property, and also wildlife-to-wildlife transmission. The epidemiology of MAP infection and transmission is very complex, and wildlife disease reservoirs only increase this. Rabbits have been shown to be asymptomatic carriers of MAP, and shed such significantly high levels of MAP that one faecal pellet may present an infectious dose of bacteria for cattle (Daniels *et al*., 2003). Cattle do not show any behavioural avoidance of rabbit faeces when grazing (Judge *et al*., 2005). Rabbits tested on farms with a low response to control measures had a greater probability of being found to be MAP-infected (0.4) than those on farms who were responding to interventions (0.1), indicating that rabbits are a real reservoir of infection for cattle (Shaughnessy *et al*., 2013). To combat this risk of infection, a reduction in the wildlife population could be

undertaken depending on the type of infection present. Davidson *et al*. (2009) investigated this using a stochastic compartmental model where individuals could move between the two disease states; susceptible or infected. Other states were also present, such as age, birth and death. They found that on-off culls removing individuals would have to remove over 96 % of rabbits and on-off removal of social groups needed cull levels of over 98 % before any effects on disease control are seen. Repeated annual culling was the more effective option, as only 45 % of the population had to be removed to gain eradication. However, even if culling of the local rabbit population was undertaken, the herd would still be at risk of infection from other wildlife. For instance Beard *et al*. (2001) isolated MAP from foxes, stoats, weasels, crows, rooks, jackdaws, rats, wood mice, hares and badgers. Cetinkaya *et al*. (1997) concurred when they studied mixed enterprises, finding that farms with farmed deer were over fifteen times more likely to report disease than those without (odds ratio 15.2 to 209.3). In addition, MAP has previously been isolated from wild ruminant populations such as deer (Ryan *et al*., 2006).

1.3.3 Disease Control

1.3.3.1 Management

Managing MAP infected herds can be achieved through multiple basic strategies; preventing new infections through biosecurity, hygiene and management, managing infected animals through test-and-manage/cull programmes, and improving resistance with vaccination and genetic selection.

According to Sweeney *et al*. (2012), these three approaches can be implemented at varying levels depending on how aggressively the farm wants to tackle the disease, i.e. whether their goal is stabilisation, reduction or eradication. However, control programmes should be herd-specific and practical for those taking part and need to take into account the strengths and weaknesses of an individual farms, including infrastructure and staff members ability and knowledge. Any strategy needs the full commitment of the farmer and other staff as realistic time lines for eradication are long (10+ years), so it can be easy for farmers to become unengaged with the required practices if benefits are not seen quickly (Garcia & Shalloo, 2015; Geraghty *et al*., 2014; Orpin & Sibley, 2014; Ritter *et al*., 2015).

Looking in detail at the elements of a control programme described above, prevention of new infections is the most important part of a control strategy, as this will stop the spread of disease to a new generation (Ridge *et al*., 2010). The most susceptible group within a herd are new-born calves and young stock (Mortier *et al*., 2015). It is well accepted that the first few days of life are key for transmission, however Mortier *et al*. (2013) conducted an oral inoculation trial where 56 calves from MAP-negative dams were challenged at either two weeks, three, six, nine, or twelve months. All calves were then euthanized at seventeen months of age and culture undertaken on numerous tissue samples. Calves from all age groups were infected, confirming that good calf hygiene should not end when the calves have been separated from their mother. Eisenberg *et al*. (2015) describe the first few days of life as an important moment for transmission in their survey of 117 dam-daughter pairs

where faeces, colostrum and environmental samples were collected. They concluded that if calves are raised in a contaminated environment, the disease status of the dam is not a significant factor, again highlighting the importance of a clean calving environment.

The first thing in the calving cycle that can be done to reduce disease burden in the herd is breeding all animals to a terminal sire, which is a practice recommended by National Action Johne's Group (2017) in their campaign to farmers titled 'Know your Johne's status and how to control it' (National Action Johne's Group, 2014). This involves serving all dairy cows with beef semen and the resulting calves fattened for sale, meaning that no replacement heifers are taken from infected cattle. However, this practice could put the herd at risk in itself as there is a possibility of buying in more infected cattle as replacements, so many farms choose to just serve animals who are identified as JD positive to beef. Most farms choose to calve all JD positive cattle separately to negatives, as this reduces the risk of accidental ingestion of MAP, for the dams own calf and others.

The aim of hygienic rearing practices is to break the transmission cycle of MAP by removing calves from their dams as quickly as possible, ideally within 24 hours (Windsor & Whittington, 2010), and segregating them away from the rest of the herd until they are over twelve months of age. Windsor & Whittington (2010) conducted a review of studies relating to calf rearing practices, finding that there was no direct evidence to support the removal of calves within twelve hours, which is the commonly accepted timeframe.

However, if exposure were to occur at birth, then the risk of infection progressing to JD is very high, especially in a highly contaminated environment or if the dam is infected. This suggests that it is not worth snatch calving (removal directly after birth) all animals within a herd, but rather those who are infected, reducing the burden on herdsmen making it easier to maintain a control programme. If the farm does not have the facilities to segregate calves or is very heavily infected, it has been suggested by Aly *et al*. (2015) that off-site rearing can reduce the number of animals that seroconvert and shed MAP in their faeces and decrease JD related culling up to six years old, especially if they are reared off-site from day one to just before calving.

Even if the most important route of transmission (faecal-oral route) has been removed, a calf is still at-risk after removal from the dam. Clean colostrum and milk from a non-infected cow are also very important, and not just for JD transmission, as many farms will feed milk from cows with a high somatic cell count or on antibiotics which can increase the risk of antimicrobial resistance within the herd. Nielsen *et al*. (2008), using information from 808 farms enrolled on a Danish control programme, found that calves fed colostrum from multiple cows were more likely to become ab-ELISA positive than those fed from their dam only (odds ratio 1.24). Calves were also more likely to become ab-ELISA positive if they had suckled from a foster mother compared to those fed on milk replacer (odds ratio 2.01). With regards to feeding milk with high somatic cell count, this did not increase the risk of being JD ab-ELISA positive. This study shows that milk is a risk factor for transmission of MAP,

but the source of this milk, be it pooled or from the dam, is insignificant as long as it is clean.

In an attempt to improve the quality and safety of the milk fed to calves, many farms pasteurise colostrum and waste milk before feeding. As mentioned previously, the feeding of waste milk to calves is discouraged due to concerns with bacterial contamination and transmission of disease (Elizondo-Salazar *et al*., 2010). However, many farms still do this as it utilises something that would otherwise go to waste. Elizondo-Salazar *et al*. (2010) evaluated pasteurisation on six dairy farms in Pennsylvania and found that this process is very effective at lowering the bacterial contamination of the milk. Stabel (2001) first conducted an evaluation of experimentally inoculated waste milk, which was then heated at 65.5 °C for 30 min, finding that no viable bacteria could be recovered after 28 weeks of incubation on Herrold's egg yolk medium (HEYM) at 37 °C. This implied that on-farm pasteurisation efficiently inactivated MAP. The same group followed up this study, using high-temperature, short-time pasteurisation (64 °C to 71.7 °C for 15 s) for colostrum samples inoculated with three different strains of MAP. In this study MAP was found to be increasingly sensitive to pasteurisation as the temperature increased, however it was also found that increasing temperature affected the concentration of the milk immunoglobulin G suffered, so reducing its overall protective quality (Stabel *et al*., 2004). Again, Stabel (2008) extended these findings by investigating the benefits of feeding pasteurised colostrum to calves from dams who had been naturally infected

with MAP. Calves were split into two groups, where they were either fed with colostrum from their own dam, or fed pooled pasteurised colostrum from healthy dams. Although calves from both groups were found to be positive for MAP in multiple tissues sites when culled at 12 months old, less MAP positive animals were found in the group fed from pooled colostrum, indicating that clean colostrum from MAP free dams may decrease initial exposure to the bacterium. However, this result may have been confounded, and the true result even better than presented as at six weeks of age all of the calves were weaned and housed together for the next twelve-month period. This gave ample opportunity for further horizontal infection from calves fed colostrum from their own dam shedding in the faeces as described by van Roermund *et al*. (2007) and discussed previously. However an extended five year study of calves fed pasteurised pooled colostrum compared to non-pasteurised, was carried out by Godden *et al*. (2015) which noted that after the prolonged period there was no difference in the proportion of animals testing positive for MAP when they compared those fed heat treated colostrum to fresh colostrum.

Another of the management strategies identified by National Action Johne's Group (2014) is test and cull, which they suggest is suitable for low prevalence herds. This aims to remove infected animals from the herd as soon as they are identified, and to work alongside other management practices. Lu *et al*. (2008) modelled this approach to control by using the R_0 and comparing good and poor herd management (low and high transmission rates, respectively). As expected, culling only high shedding animals detected by faecal culture in a

herd with good management was predicted to be effective at controlling MAP transmission. However, faecal culture is not widely used in control programmes, and ab-ELISA does not necessarily identify those animals who are shedding MAP in the faeces (Gao *et al*., 2009). In the case of poor management, culling low-shedding animals was predicted to also be required to control transmission.

1.3.3.2 Treatment and vaccination

Although there is no known definitive cure for JD, there may be several therapeutic strategies that can be applied to reduce clinical signs and also prolong the life of an infected cow, but these unfortunately do not stop shedding (Fecteau & Whitlock, 2011) and involve the use of high cost drugs and daily therapy (Emery & Whittington, 2004). Fecteau & Whitlock (2011) conducted a review of treatments, discussing multiple antimicrobials that have been used in experimental and natural cases, including isoniazid, rifampin, clofazimine, aminoglycosides and dapsone, which are better when used in combinations. All of these are deemed to be more effective than other therapies as they are able to penetrate mammalian cells; for example, rifampin enters leukocytes. They also reviewed the use of monensin as a prophylactic, fed to neonatal calves at the point of high susceptibility, and it was found that calves fed this drug had fewer faecal culture positive test results than the control group. However, Emery & Whittington (2004) describe antimicrobial therapy as impractical and economically unjustifiable, with treatment for an average 50 cow infected herd costing $2472 (£1976)

annually (Chi *et al*., 2002), and the animal will not be able to be used for food consumption. Interestingly, they also suggest bacteriophage as an alternative therapy due to the rise of antimicrobial resistance.

The aim of vaccination is to control the cycle of infection, with some evidence that it can reduce the incidence of clinical disease (Emery & Whittington, 2004). Firebreak vaccination is the sixth suggested strategy by the National Action Johne's Group (2014), explaining that it may be a short term option for high risk, high prevalence herds to 'buy some time' until another strategy can be implemented. The only issue with this is that it will then become very difficult to differentiate between antibodies from vaccination and infection, unless a DIVA test (Differentiating Infected from Vaccinated Animals) can be used, of which none are currently available. Vaccination of a herd can be achieved with live-attenuated or killed vaccines. In the UK only one vaccine is licenced for use (Gudair, CZ Veterinaria/ Virbac), and this can only be used for vaccination of sheep and goats (APHA, 2017). One reason for this is that JD vaccination has been shown to have some cross-reactivity with the bTB antigens used in the skin test for *Mycobacterium bovis* infections (Coad *et al*., 2013). Groenendaal *et al*. (2015) add that JD vaccination could cause additional indirect costs to a national bTB control programme, estimating that this could be upwards of $8.03 (£6.42) per cow per year to dairy producers. This would be especially important in the UK, where the national bTB programme already costs the government £100 million annually in testing and compensation for lost stock (Defra, 2016).

1.3.3.3 National control schemes

Many countries now have national voluntary control programmes, with the UK being no exception. The National Johne's Management plan has been developed by The National Action Johne's Group (2017) to help provide strategies and training to veterinarians to manage the control of JD on farm. This action group is industry led, supported by milk purchasers, testing laboratories, farming unions, breed societies, government bodies and veterinary associations. This management plan is currently in phase two, where they are seeking views from industry on how to tackle the disease, the previous phase focused on education and engagement of veterinarians and farmers. However, this programme is still in early stages compared to similar schemes in other countries (Geraghty *et al*., 2014). The long-term aim of the UK programme is to get farmers to subscribe to at least one of the six control strategies outlined in Table 1.1, which are thought to cover all dairy farming scenarios. These variations have been developed since, as previously mentioned, a control programme needs to be appropriate for an individual farm.

Table 1.1 Six strategies identified by the National Action Johne's Group

Strategy		Description
1	Biosecurity: protect and monitor	Proven negative herds. A robust biosecurity and surveillance protocol established to protect the herd.
2	Improved farm management	Low risk, low prevalence herds. Control by breaking the cycle of infection by changing management practices.
3	Improved farm management: strategic testing	Higher prevalence herds. Identify cows earlier through strategic testing.
4	Improved farm management: test and cull	Low prevalence herds. Removal of infected animals quickly before they get the chance to spread JD
5	Breed to terminal sire	High risk, high prevalence herds. Replacements sourced from herds with low JD risk, all calves fattened for slaughter and must not enter a herd for breeding.
6	Firebreak vaccination	Short term option for high risk, high prevalence herds. Gives a short break until another strategy can be adopted.

The National Action Johne's Group provides farms who sign up to the programme six control strategies they can adopt depending on their prevalence and other management procedures.

Apart from management, the main backbone to each strategy is testing, with all farms encouraged to sign up to a suitable testing regime. In the UK, the National Milk Laboratories (NML) offers two levels of testing; herd, where targeted cows or bulk tank tests are conducted, or cow, a quarterly test of every milking animal. Their recommended herd-level testing option is a 30-cow milk ab-ELISA screen or blood serum ab-ELISA screen for beef herds (National Milk Laboratory, 2017), which targets animals most likely to show a positive test result, such as those that are lame, mastitic or with a chronic high somatic cell count. The effectiveness of this approach was compared to random sampling of 30 cows by Hanks *et al*. (2014), who found that targeted selection of animals improves the likelihood of identifying a positive cow, from 84 % in a random sample to 95 % in the targeted samples. This likelihood of finding a test-positive animal with targeted testing is seen in all levels of within-herd prevalence, and most dramatic in low-prevalence herds (Fig. 1.7). However, bulk tank tests for identifying infected herds are not recommended due to dilution effects where a relatively high number of animals could have antibodies in their milk before the test would reflect this (National Milk Laboratory, 2017). Cow-level testing is also carried out as part of the Herdwise scheme which is arranged in collaboration with National Milk Records (NMR; 2017). In this case every cow is tested on a quarterly basis using the milk recording samples. These results are then used to generate a longitudinal test profile for each cow, which can be classified into a risk group (Table 1.2). A similar scheme and risk rating system is also used by other milk testing

companies, such as the Cattle Information Service (CIS) and Quality Milk Management Service (QMMS).

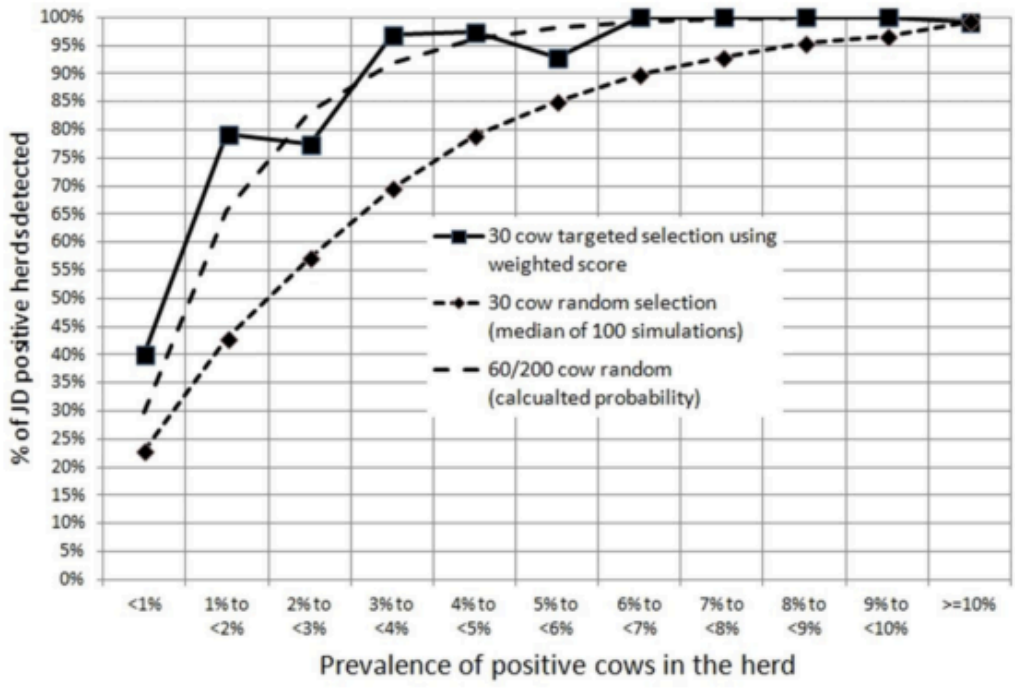
All these testing schemes are accredited by the Cattle Health Certification Standards (CHeCS), which also supports the National Johne's Management plan. CHeCS is the regulatory body for cattle health schemes in the UK and Ireland, providing standards and certification for health schemes and promoting cattle health and welfare (CHeCS, 2017). They also provide certification to any herd who meets the agreed national health standards, providing a certification goal to those who strive for eradication and helping to create an incentive for farmers to remain engaged with the process.

Table 1.2. Johne's risk group as defined by commercial testing companies

Johne's Risk Group	Definition	Risk Level
J0	Repeat ab-ELISA negative (minimum two tests)	Low
J1	ab-ELISA negative but only one test (first lactation heifer or bought in stock)	
J2	ab-ELISA negative but positive within three previous tests	
J3	ab-ELISA positive/negative interchangeably	High
J4	Last ab-ELISA test positive, all previous tests negative	
J5	Repeat ab-ELISA positive (minimum two tests)	

This information is supplied by National Milk Records to farmers to help them make management and culling decisions on their cattle based on the previous three ab-ELISA test results, where ab-ELISA negative cows are defined as having an OD $_{600nm}$ <20.00 and those >30.00 being classed as positive

Figure 1.7. Comparison of targeted selection against random sampling of 30 and 60 cows for detecting positive herds at different prevalence levels



Targeting sick or 'poor-doers' was proven to be very effective at identifying MAP-infection within a herd (solid line), with a higher percentage of cows identified with this method than random selection of cows (dotted line) (taken from Hanks *et al*. (2014)

One of the largest challenges is recruiting farmers to voluntary control programmes, despite extensive communication, outreach activities and low cost. To understand this better, Ritter *et al*. (2015) investigated the differences between the farms who enrolled on to the Alberta Johne's Disease Initiative and the 35 % of those who did not enrol. They used a questionnaire which included demographic characteristics, internal factors such as attitudes and beliefs, and external factors such as farmers knowledge and on-farm constraints. They found that farms who did participate in the initiative tended to be larger herds, with the farmer having a higher self-assessed knowledge of JD, better understanding of the details surrounding the initiative, and they often used their vet to get up-to-date information on management practices. Within the non-participant group, time was reported as being a major restrictive factor, with farmers stating that they felt that participation would take up too much of their time. Inertial or a lack of confidence in the scheme also seem to be a factor as many of them indicated that they would like to wait and see how the programme worked on other farms before they participated. A similar study was conducted by Nielsen (2011) using the Danish voluntary, producer-paid control programme, where approximately 28 % of herds within the country were enrolled. They found that the majority of farmers joined the scheme because they wanted to achieve disease control and gain accreditation, along with the incentive of being able to avoid production losses and an awareness of the need to increase consumer safety. Increasingly, participation in JD control programmes has been written into UK farmers milk contracts, with producers such as the Tesco Sustainable Dairy

Group removing farms from their pool of milk suppliers if they are not involved in a disease control initiative (James, 2017). In addition, smaller food producers such as Barbers Farmhouse Cheesemakers, are also promoting JD programs by giving farmers who engage with a control plan a 1.5p/litre bonus (James, 2017). Overall, it is in a farms best interest to take part in a control scheme as they will have access to tailored advice and possible bonuses from food processors.

1.3.4 Disease control modelling

Field-based studies on JD prevalence, incidence and control measures are difficult to conduct due to cost, time required for clinical disease to develop and limitations of existing tests. Therefore, stochastic simulation models are very useful for estimating transmission rates and other parameters. Early attempts to model the epidemiology of JD were highly theoretical and parameterisation was not considered in depth (Collins & Morgan, 1991), making the results generated unreliable. Others have used published literature and opinions to parameterise the models, but this does not account for the between farm differences known to exist (Groenendaal *et al*., 2002; Pouillot *et al*., 2004). Many models have been designed to simulate JD control measures, such as culling, hygiene management and vaccination. Despite their limitations, simulation models have become a very fashionable way of studying control programmes especially as we can now overcome the above downfalls. Groenendaal *et al*. (2002) developed a stochastic and dynamic

simulation model to evaluate the change in disease patterns when control strategies were implemented alongside the economic consequences of these strategies. They used a mixture of expert opinion and a literature review to parameterise the model. They found that calf hygiene was a key control measure, with annual losses doubling if this was ignored, whereas test and cull strategies only had a small effect on disease prevalence. However, only one herd was represented within this model, which ignores the environmental differences that exist between farms, limiting its wider applicability for the industry. Like Groenendaal *et al*. (2002), Pouillot *et al*. (2004) used parameters provided by a panel of experts within a deterministic and stochastic model. Using this they found that incidence rates predicted were in line with values published in the literature, at 21.2 % of animals in a dairy herd. However model validation was difficult to achieve as infected herds had been poorly monitored in France and there are only a few field surveys available. Further investigation by Lu *et al*. (2010) showed that MAP infection could persist within a herd for more than 20 years if an annual test and cull protocol was implemented. However, MAP infection would become extinct within this time period if a biannual protocol was observed.

Since then, newer techniques have emerged to allow for uncertainty in parameter estimation to be taken into account. Davidson *et al*. (2012) developed a new technique for parameterising models which possibly provides one of the best representations of the disease to date. This works by using an agent based stochastic model that uses a novel reweighted Latin hypercube sampling (LHS) scheme for parameterisation. This means that the

infection state and history of each animal is stored within the model, making test results depend on how long the animal has been infected. Parameterisation is used to give values to unknown or variable qualities in the model, with shedding rates of infected animals and the survival of bacteria within the soil as prime examples. LHS parameterises the model whilst accounting for the fact that it cannot be done with much precision and that it will differ between farms and herds. It does this by generating a score for each of parameter values, based on how well the model recreates a set of real data, then combining results from each to generate an overall probability for each set of parameters. Using these methods, differences between farms and parameter uncertainty can be accounted for, which is extremely important when small changes in these values can give very different outcomes.

Finally, the use of Bayesian statistics has become popular in the last few years, where prior information or knowledge is used to accept or reject hypotheses. This is particularly useful when a perfect reference standard is not available to compare to, or the Gold Standard detection is imperfect (Wang *et al*., 2011). This method has also been used to estimate herd- and animal-level true prevalence in Ireland by McAloon *et al*. (2016) who analysed records of approximately 99,000 animals from 1039 dairy herds which were generated as part of the national control programme. Prior information and assumptions were taken from Nielsen & Toft (2008) on case definitions and estimated Se and Sp of the commercial testing kit, and Good *et al*. (2009) for details on observed prevalence in Irish herds. Using this information the estimate that herd-level true prevalence (i.e. the probability of a randomly selected herd

containing at least one true positive animal) was 0.28 and the animal-level found to be 0.032.

Modelling of disease progression and control programmes can provide a cost-effective alternative to large field trials, and are becoming more accurate as new techniques such as those discussed above emerge.

1.4 ZOONOSIS

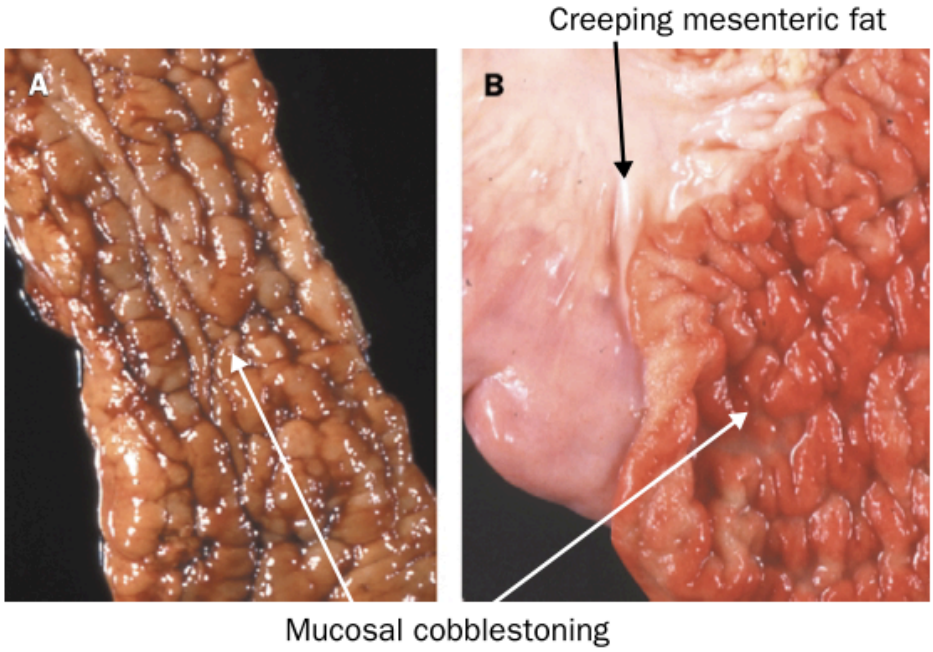
MAP is thought to have a potential role in Crohn's disease (CD), an inflammatory bowel condition with similar pathological changes to JD (Fig. 1.8), thought to be auto-immune in origin with increasing evidence pointing to an infectious agent triggering the disease (Greenstein, 2003). MAP is also reported to be present in co-infections in cystic fibrosis patients (Olivier *et al*., 2003), Although the latter is more opportunistic in nature, these links makes it a bacterium of potential zoonotic importance.

1.4.1 Crohn's disease

There has been a significant increase in incidence of CD in the developed world over the last 50 years, and levels appear to be increasing along with progressive industrialisation in less-developed countries (Shanahan, 2002). Without a suitable model it is hard to provide evidence for Koch's postulates, with MAP being isolated from tissue biopsies of both cases and controls (Bull *et al*., 2003; Scanu *et al*., 2007). However, a meta-analysis conducted by Feller *et al*. (2007), where 28 publications comparing CD cases with disease-free controls were reviewed. They identified an association between the presence of MAP and development of CD. Eighteen of the studies contained in the meta-analysis used PCR to identify MAP DNA from biopsies, with the analysis finding that the prevalence of MAP was 7.01 times higher in patients with CD than in controls within sixteen of the eighteen studies. When ab-ELISA tests were considered (thirteen studies), like PCR, the prevalence for MAP

antibodies was higher in patients with CD than in controls in ten of the thirteen studies, with an odd's ratio of 1.72. On this basis, they concluded that CD patients were more likely to be MAP test positive than those either disease free or ulcerative colitis sufferers. However, it must be noted that cause and effect is still not proven.

Figure 1.8. Comparison of intestine from a JD infected cow and a CD patient



The mucosal membrane of the ilium in a CD patient (A) compared to a JD positive cow (B), showing the similarity between gross pathology. Taken from Greenstein (2003).

1.4.2 Pasteurisation

Cattle infected with MAP shed substantial levels of the bacteria into their milk, faeces and semen (Antognoli *et al*., 2008), with milk being highlighted as a key transmission vehicle for human exposure to MAP. This makes the efficiency of pasteurisation an important factor in control of transmission. Pasteurisation has played a role in the decontamination and shelf-life extension of milk for many years (Holsinger *et al*., 1997), and was first coined by Louis Pasteur in the 1860's during experiments on wine and beer (Steele, 2000). Early commercial pasteurisation was not widely adopted, but as the incidence of several milk-borne diseases such as typhoid fever, brucellosis, diphtheria, tuberculosis and anthrax were increasing, the uptake of the process increased as they recognised the advantages of this in improving human health. The first piece of legislation defining the process was passed in 1924, where it was defined as a heating process of not less than 61.1 °C for 30 min in approved equipment, dubbed the 'holder method' or low-temperature long-time (LTLT; Slana *et al*., 2008). Research continued, and high-temperature short-time pasteurisation (HTST) was introduced following the development of equipment design allowing more rapid heating and cooling (Holsinger *et al*., 1997). During HTST pasteurisation the milk is heated to 71.1 °C for 15 s which has been proved to be more efficient at destroying bacteria than other methods (Chiodini & Hermon-Taylor, 1993; Grant *et al*., 1998). However neither method was totally reliable (Steele, 2000) with many studies since then reporting various levels of MAP survival in milk.

Multiple studies have found that neither LTLT nor HTST pasteurisation totally inactivated MAP when it is present at levels over 10^4 cfu ml^{-1} (Chiodini & Hermon-Taylor, 1993; Grant *et al*., 1996a). As a follow-up to this finding, Grant *et al*. (1998) found that MAP could only be reliably inactivated from laboratory inoculated milk at levels of 10 cfu ml^{-1} or 10 cfu per 50 ml, and was present in 14% of all other samples containing levels higher than 10^2 cfu ml^{-1}. This finding led to most dairy processing centres adjusting the holding time from 15 s, which was the legal minimum, to 25 s therefore increasing the likelihood that complete inactivation of MAP would be achieved.

MAP has been cultured on HEYM from 1.8 % and detected by PCR in 11.8 % of pasteurised milk from commercial milk pasteurisation plants across the UK (Grant *et al*., 2002), with three-quarters of the culture positive samples coming from dairies still using the 15 s holding time. Since then O'Reilly *et al*. (2004) conducted a similar survey on approved Irish pasteurisation plants, again using HEYM and PCR. They found that 12.9 % of samples contained MAP detectable by PCR, and no samples contained MAP detectable by culture. They concluded that MAP is only occasionally present at low levels and pasteurisation procedures were then considered effective. Other studies have been conducted outside of the UK, in the Czech Republic (1.6 %; Ayele *et al*., 2005), United States (2.8 %; Ellingson *et al*., 2005), and Argentina (2.86 %; Paolicchi *et al*., 2012), amongst others, all detected by culture on HEYM. To this date there have been no further published studies on the prevalence of MAP in pasteurised milk in the UK.

The presence of MAP in low levels within pasteurised milk may explain why it can be isolated from both cases and controls in studies of CD, due to the large amount of milk consumed in the developed world. However, Feller *et al*. (2007) suggest that there are concerns that the use of a more thorough heat treatment, which would eliminate MAP, would change the organoleptic qualities of milk and adversely affect its taste. Due to the association made by Feller *et al*. (2007) between MAP and CD, milk processors and supermarkets are already trying to enforce JD management and testing, with some even cutting prices if these are not implemented (James, 2017).

1.5 DIAGNOSTICS AND DETECTION

Research into JD has been severely hampered by the slow growth of MAP, which can take up to 18 weeks to form visible colonies on solid culture (Rajeev *et al*., 2006). In turn, this can make both diagnosis and detection of JD difficult and time consuming. The two main tests used for diagnosis of JD are faecal culture (considered to be the Gold Standard) and ELISA for the detection of antibody in blood and milk. The latter is the most commonly used test in control programmes. These two tests measure different aspects of the disease; the shedding of live bacteria into the gut lumen and an indirect antibody response to infection, respectively. Testing in parallel - conducting multiple tests and diagnosing an animal as positive if one (not all) of the tests is positive – increases the chance false positives due to the decrease in Se caused by testing in this way. In addition, there is a potential for passive shedding, whereby the cow is shedding bacteria in faeces due to ingestion of the organism but is not infected (Kralik *et al*., 2014). In theory, the Sp of faecal culture would be 100 % as an bacteria can only be cultured if they are present in the sample. However, due to passive shedding, Sp decreases to approximately 98 % (Espejo et al., 2015). Statistically, there is a significant drawback to testing entire herds because of the poor positive predictive value of the tests, caused by the greater likelihood of a false positive result in a truly negative herd when large numbers of animals are tested. In contrast to this, diagnostic testing of individual animals with a low pretest probability of infection is correlated with poor negative predictive value (Harris & Barletta, 2001). As it stands, all detection methods currently in use are imperfect, so no

matter what is used, or how it is used, all infected animals are not captured. The ultimate research aim is to have a tool that can be used to discover more about the disease, then in turn used to develop focused control plans.

The poor performance of detection methods has an economic effect on the industry, as highlighted by Kudahl *et al*. (2007). They used simulations to study test and cull control measures, where a cow is culled as soon as it become positive, comparing the current test parameters to one with an improved Se. An improved ab-ELISA was predicted to reduce the prevalence of JD more effectively than the current parameters, with milk production increasing up to the level of a non-infected herd within ten years. However, the improved test would be more expensive than the current protocol, due to the increased rate of replacement cattle needed to compensate for the increased number of cattle lost.

1.5.1 Culture

The Sp of any culture method is usually assumed to be 100 %, as for MAP to grow it would have to have been present in the original sample (Nielsen & Toft, 2008). Detection of viable MAP in milk or faeces with culture involves chemical decontamination to remove other competitive bacteria, such as *Escherichia coli* which is commonly found in these matrices. However, this can also cause a decrease in the number of viable MAP in the sample (Dundee *et al*., 2001; Grant & Rowe, 2004), sometimes to quite a significant level, of up to 4 $\log_{10}$ (Dundee *et al*., 2001), which means that the number of cows detected

is limited to those shedding high levels of MAP. The Se of culture as a diagnostic can be between 5-70 % depending on the method used, with Whitlock *et al*. (2000) stating that faecal culture had a Se of 38 % for one-off testing, but increased to 42 % when tested on multiple intervals.

There are a number of culture methods available, with incubation on solid slopes of HEYM being the most common. Multiple automated rapid culture methods are used in commercial laboratories. BACTEC tubes measure the release of ^{14}C-labelled CO_2 to indicate the growth of mycobacteria in liquid culture (Williams-Bouyer *et al*., 2000). MGIT has replaced BACTEC in recent years (Kawaji *et al*., 2014). ESP Trek monitors liquid culture for a decrease in oxygen, and will fluoresce if there is growth (Williams-Bouyer *et al*., 2000).

Long incubation periods of up to 18 weeks follow the initial decontamination step to remove fast growing bacteria, and then further molecular identification tests are required to confirm that MAP DNA was present in the original sample (Eamens *et al*., 2000). The extremely slow growth of MAP is partially attributed to its inability to produce mycobactin; a siderophore that is responsible for the binding and transport of iron into cells (Batt & Guscetti, 2009). Due to this need, Mycobactin J must be included in any media used to culture this bacterium. This requirement for multiple tools, increased man-hours and specialist media make culture the most expensive testing option a farmer can use, with commercial laboratories charging around £42 per sample (SAC consulting, 2016). Due to the decontamination steps required, all of these methods have limited Se, therefore making it difficult to accurately

detect the presence of MAP. Despite this, faecal culture is considered the Gold Standard diagnostic method pre-mortem.

Many studies have found that results gained using culture and other diagnostic tests do not correlate. For instance Stabel *et al*. (2002) found that only 25 % of faecal culture positive cows also gave a positive test result with ab-ELISA and PCR, with 6 % of culture negative animals being MAP-positive using ab-ELISA and PCR. Gao *et al*. (2009) also reported this issue of discordance between test results, finding that there was a significant relationship between milk culture, direct PCR and nested PCR, but that faecal culture results were not related to any of the other assays.

1.5.2 Immunological tests

1.5.2.1 Enzyme-linked immunosorbent assay

ELISA can be used for the detection of MAP with various sample types including milk and blood. Johne's disease specific ELISAs are designed to detect MAP specific antibodies and so the animal's immune response to infection or exposure to the organism. They are low cost to the end-user and are relatively high through-put, making them a popular diagnostic tool. From 1990 to 2000 the estimates of the Se of the milk ab-ELISA assay was dropped from 57 % to 45 % (Whitlock *et al*., 2000), and this was shown to increase as age of the animal increased due to the progression of sub-clinical disease into clinical and advanced stages. Nielsen *et al*. (2013) showed that at two years of

age the Se of the ID Screen® Paratuberculosis ELISA test (ID Vet, Montpellier, France) was estimated to be 27 %, increasing to 54 % at three years and 68 % at four years of age with the maximum Se of the test reaching 79 % at ten years of age. However, this was using the criteria that one positive test result defined a case of infection. If this criterion was increased to two positive test results the Se was found to be only 18 %, although the test had a similar high Sp to other tests at 98 %. Overall, reported test sensitivities range from 7 % (McKenna *et al*., 2005) to 45 % (Collins *et al*., 2005) for blood ab-ELISAs and 6 % to 65 % for milk ab-ELISAs (Nielsen, 2010). There has also been some variability in ab-ELISA results reported depending on the stage of lactation of the cow, with a cow more likely to yield a positive test result at the beginning of its lactation using the milk ab-ELISA and at the end of lactation using the blood ab-ELISA (Nielsen *et al*., 2002).

However, one issue that is a particular problem with the use of ab-ELISA in UK herds is cross reaction with bTB and the single intradermal comparative cervical test (SICCT), which is a simple diagnostic method for bTB involving an injection of *M. bovis* and *M. avium* tuberculins under the skin. Kennedy *et al*. (2014) investigated this by serum and milk sampling a herd of 139 cows with minimal bTB infection, pre- and post-SICCT. Prior to SICCT testing, 7.9 % of cattle gave a positive JD ab-ELISA test result in serum, and 5.8 % in milk. This increased significantly post-SICCT, with the highest recorded prevalence for both serum and milk samples being 39 %. There was a significant difference between pre- and post-SICCT milk ab-ELISAs up to 43 days after the skin test was administered, with this increasing to 71 days for the serum ab-ELISA.

However, the increase in test-positive animals was not significant until up to 72 h post-SICCT.

To take into account the cross-reactivity with other mycobacteria and the general immune response from exposure to MAP, an optical density (OD) threshold is used. For MAP detection, an animal with an OD of over 20 is inconclusive and any over 30 is positive. These thresholds can be adjusted depending on the Se and Sp needed for a control programme, with the removal of the thresholds increasing the number of false-positive animals.

Even though ab-ELISA has its downfalls, Roussel *et al*. (2007) explain that as it is inexpensive at under £5 per test, and is widely available at all veterinary diagnostic laboratories and milk recording companies, it is sufficient to be used as a screening tool.

1.5.2.2 Interferon gamma

Like the ab-ELISA tests, the IFN-γ test detects an immune response against the bacteria. IFN-γ is released by the immune system during early infection, with several studies showing that when mycobacteria are successfully phagocytosed by the macrophages they have been pre-activated by IFN-γ (Coussens, 2001). For the diagnosis of MAP the assay has a Se of 13 - 85 % and a Sp of over 83 %, however Jungersen *et al*. (2002) warn that interpretation of the test must be adjusted to fit specific needs and the context in which the test is being applied as a large number of false-positives were observed when the test was applied to calves under 15 months old.

1.5.3 Polymerase chain reaction

PCR is a rapid and commonly used method for the detection of MAP. The discovery of several species-specific signature sequences allowed specific identification of MAP, with IS*900* and *f57* being those commonly used (Slana 2008). IS*900* is the accepted standard signature sequence for MAP identification and detection, but some PCR assays which target this sequence have been shown to generate false-positives (Englund *et al*., 2002). Despite this they are favoured because other elements do not provide as high Se as the multicopy IS*900*, so this remains the standard signature sequence (Slana *et al*., 2008).

To prevent inhibition of the PCR reaction, preparatory steps are needed to isolate the cells from the sample matrix, such as centrifugation or immunomagnetic separation prior to DNA extraction (Grant *et al*., 2000). The Se of PCR is reported to be 4 - 76 %, depending on the microbial load and sample matrix, but provides a Sp of over 99 %. Using a PCR assay, Stabel *et al*. (2002) found that 68 % of the milk samples tested were MAP-positive, whereas all samples were negative when cultured. This discrepancy could be either due to a difference in the Se of the two methods or because PCR cannot differentiate between live and dead cells.

1.6 PHAGE-PCR

The *FASTPlaque*TB assay was first established to rapidly detect *Mycobacterium tuberculosis* in human sputum samples in developing countries where expensive laboratory equipment was not accessible. This assay, now known as phage-PCR (PBD Biotech Ltd, 2017), directly detects viable mycobacteria by the ability of the mycobacteria contained within the sample to protect and support the replication of mycobacteriophage D29. The phage used has a broad host range, infecting all mycobacteria, therefore the phage element is combined with other methods to provide Sp. It is achieved via PCR identification of DNA from the original cell present in the plaques formed at the end of the phage assay as DNA from the original cell is preserved in the centre (Stanley *et al*. 2007). This test has been applied to a number of sample types such as milk (Botsaris *et al*., 2013; Stanley *et al*., 2007), cheese (Botsaris *et al*., 2010) and more recently blood (Swift *et al*., 2013; Swift & Rees, 2013).

Much like traditional culture methods, this test allows the identification of viable cells only. It reduces the incubation period of standard culture from up to 18 weeks to 24 hours (a total test time of up to 48 hours when including PCR confirmation). The aggressive decontamination needed to remove fast growing bacteria from the sample is not necessary for phage-PCR. Meaning that the mycobacterium viability losses associated with traditional culture are not seen with this method (Botsaris *et al*., 2010; Stanley *et al*., 2007). Processing, such as centrifugation, is required to remove phage inhibitors

from the sample matrices, but methods have been developed that reduce the loss of mycobacteria (Botsaris *et al*., 2013; Swift & Rees, 2013). Phage-PCR also has the potential ability to be used as a DIVA test (Differentiating Infected from Vaccinated Animals), as the bacteriophage will only attach and replicate within a viable cell, giving it the advantage over ab-ELISA which cannot easily differentiate between an immune response caused by infection or vaccination, or PCR which detects all DNA, whether from dead or live cells. The Se of the phage assay applied to bulk milk samples is estimated at 90 % and Sp 99 % (Botsaris *et al*., 2013), potential making it superior to other rapid diagnostics. However further research is needed to directly compare its performance to other currently accepted detection methods.

1.7 AIM

Rapid detection of the infective agent is the cornerstone to any control programme. The detection of MAP is complicated by low Se, and multiple other issues. Phage-PCR has been used to detect MAP in a small number of raw milk samples (Stanley *et al*., 2007), bulk tank milk (Botsaris *et al*., 2013) and blood (Swift *et al*., 2013), however its performance on-farm during large scale sampling is unknown.

The main aim is to compare phage-PCR to milk ab-ELISA, which is currently used within most control programmes carried out by commercial milk testing companies, and the Gold Standard of faecal and milk culture, to investigate whether phage-PCR can detect more MAP positive animals, and provide early detection of JD. A secondary aim is to design and optimise on-farm sampling procedures to enable the assay to detect as many JD positive cattle as possible.

The final aim is to quantify the risk of human exposure through retail purchased pasteurised milk as the most recent survey was conducted over fifteen years ago using solid culture, so could be an underestimate of MAP survival in pasteurisation.

CHAPTER 2

MATERIALS AND METHODS

2.1 CULTURE MEDIA

2.1.1 Middlebrook 7H9 Broth

Middlebrook 7H9 broth (Becton Dickinson, UK) was prepared following the manufactures instructions with reverse osmosis (RO) water and autoclaved.

2.1.1.2 Media Plus

Growth supplement OADC (oleic acid, bovine albumin fraction, dextrose and catalase; Becton Dickinson, UK) and 2 mM $CaCl_2$ (Calcium Chloride; PDB Biotech, UK) was added to 7H9 broth to make Media Plus (M+) before use as a culture media. NOA antibiotic supplement (Nystatin, oxacillin and aztreonam; Mole *et al*. 2007) was also added for use with clinical samples to prevent overgrowth by non-specific bacteria and increase the number of readable results.

2.1.2 Middlebrook 7H10 Agar

Middlebrook 7H10 agar (Becton Dickinson, UK) was prepared following manufactures instructions with RO water and then autoclaved.

2.1.3 Virucide

A virucide tablet was added to 4.5 ml of sterile water following the manufactures instructions (PBD Biotech; UK). This was then left to dissolve for

one hour before use. The active ingredient within this is 10 mM ferrous ammonium sulphate.

2.2 STRAINS

2.2.1 *Mycobacterium smegmatis*

Mycobacterium smegmatis cultures were prepared by taking one colony of bacteria grown on BHI Agar and added to 100 ml M+. This was then placed in a shaking incubator for 48 h to allow the culture to reach 10^8 cfu ml^{-1}.

2.2.2 Mycobacteriophage D29

Bacteriophage were cultivated in a microtitre plate, utilising that there is approximately 1 x 10^9 pfu in a single plaque. In each well, 50 µl of agar was added, along with 10 µl of 1 x 10^3 cfu ml^{-1} *M. smegmatis*. Mycobacteriophage D29 was then added (10 µl), which combined with the *M. smegmatis* is enough to form one plaque. This was incubated overnight at 37 °C. The agar was then removed from each well, and 10 plaques put in each eppendorf tube. 1 ml of M+ was then added and the tubes placed in a shaking incubator overnight. The tubes were then centrifuged at 5,000 x g for 30 sec, and the supernatant removed. The resulting liquid was then titred, but was consistently 10^{10} pfu ml^{-1}.

2.2.3 *Mycobacterium avium* subspecies *paratuberculosis*

MAP was cultured on 7H10 (with Mycobactin J and OADC) and HEYM (Becton Dickinson, UK) and incubated at 37 °C for approximately 16 weeks. Slopes

were checked after 7 days for evidence of contamination by fast growing bacteria, and finally sealed with parafilm after colony growth. MAP strains K10, ATCC 19851 and B4 were used.

2.3 SURVEY FARMS

Table 2.1. Details of farms used within the surveys

	Breed	Average Herd Size	Milking			ab-ELISA[a]		JD Management
			Parlour type	Times	Pre-milking routine	Red[b]	Orange[c]	
Farm A	Jersey	300	36-point rotary	Twice 4:30am, 3pm	Wiped with ethanol-based disposable cloth	5	6	Snatch calving, colostrum pasteurisation, powdered milk replacer, test-and-cull[d]
Farm B	Jersey	400	20/40 herringbone	Twice 4:30am, 2:30pm	Dry wiped with paper towel	7	19	None
Farm C	Holstein-Friesian	150	12/12 herringbone	Twice 5am, 3pm	Pre-dip then wiped with paper towel	4	9	Calve JD-positive cattle separately, snatch calves from JD-positive cattle
Farm D	Holstein-Friesian	140	12/12 herringbone	Three-times 5am, 1pm, 8pm	Pre-dip then wiped with paper towel	2	4	Snatch-calve JD-positive cattle
Farm E	Holstein-Friesian	300	35-point rotary	Twice 5am, 3pm	Pre-dip then wiped with paper towel	9	17	Snatch-calve JD-positive cattle, powdered milk replacer
Farm F	Holstein-Friesian	90	8/16 herringbone	Twice 5:30am, 3:30pm	Pre-dip then wiped with paper towel	2	5	Snatch-calve JD-positive cattle
Farm Z	Holstein-Friesian	200	Robots	-	-	0	2	Pasteurisation of colostrum

a) ab-ELISA results taken from last positive test before recruitment

b) Any animal with an ab-ELISA of over 30 OD_{600nm}

c) Any animal with an ab-ELISA of over 20 OD_{600nm}

d) Removal of any animal with more than three positive ab-ELISAs

2.4 SAMPLE COLLECTION

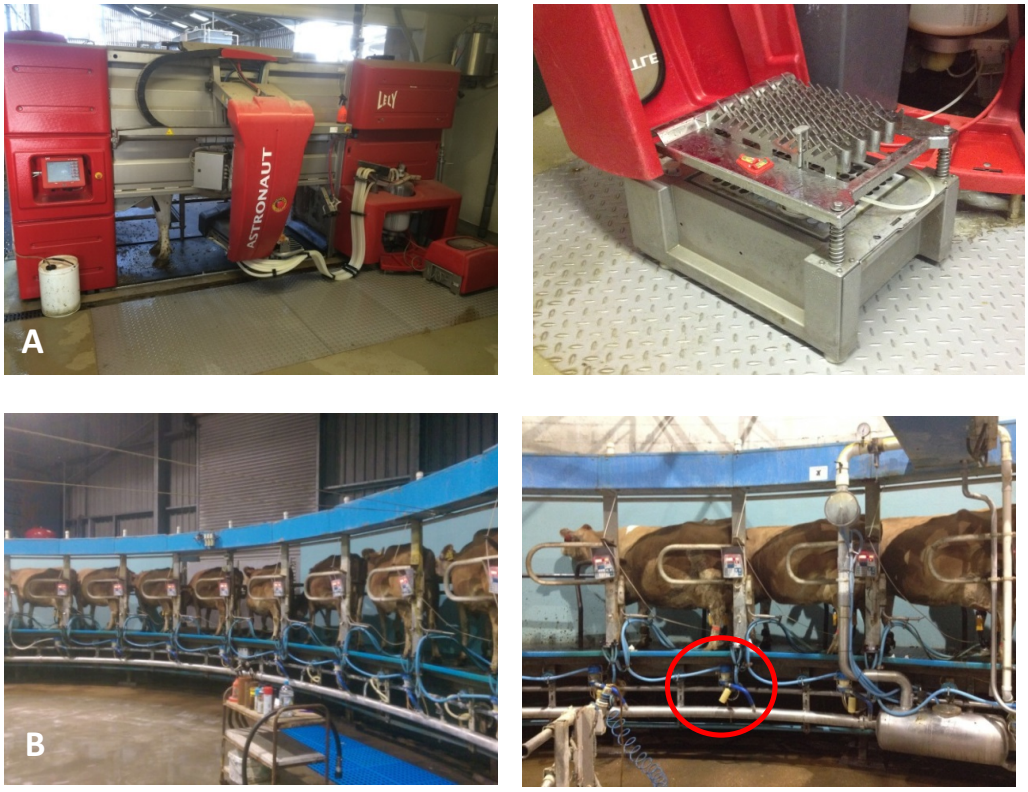
Milk samples were collected by either utilising the sample collection bottles installed in the milk line of the parlour or by hand milking of the cows in the parlour.

2.4.1 Milk sampling systems

Farms A – F are part of a monthly recording scheme, in which samples are taken to analyse the composition of the milk, alongside somatic cell counts. To do this they have a sampling collection system, usually a series of bottles connected to the milking line of each unit (Fig. 2.1 provides an example at Farm A). The bottles are under vacuum so that a proportion of milk is taken from across the whole milking. The bottles are then removed, a sample taken, and the rest discarded. These are then attached back on the milking line for the next sample.

Farm Z used milking robots rather than a parlour. Here a small machine or 'shuttle' attached to the robot after the milk meter (Fig. 2.1). A sample was taken from each cow that presented itself to be milked within the time-frame that the shuttle was set up, without taking any duplicates. This worked by milking the cow completely, releasing a portion of the milk to go to the bulk tank, taking a sample, then releasing the rest of the milk into the bulk tank.

Figure 2.1. Automatic collection systems for milk samples

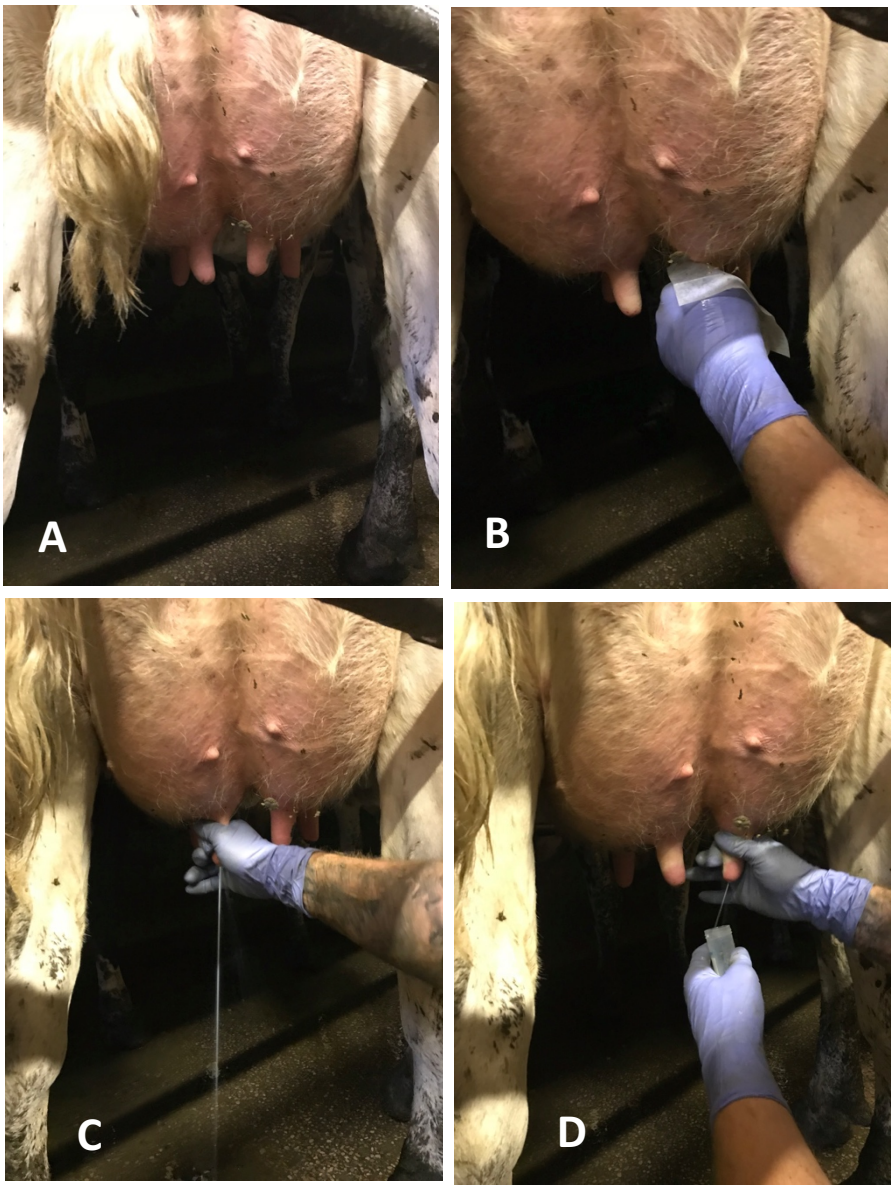


(A) represents a herd with milking robots where a shuttle was attached to the robot and took 24 ml of milk from each cow by releasing an amount from the holding jar to the bulk tank, taking a sample, then releasing the rest of the milk. (B) shows a small section of a 36-point internal rotary parlour, where the bottles attached to the milk line remove an even proportion of milk by vacuum.

2.4.2 Hand milking

With sample collection via hand milking it is very important to take a clean sample, free from faecal contamination. All farms have an established pre-milking routine, either by dry wiping, wet wiping, dipping the teats in detergent then wiping, or spraying with iodine and wiping. Samples were taken after the pre-milking routine was done. If cows were seen by the sampler to still be dirty they were wiped again using wet, alcohol-based towels, until they were fit for sampling. Each cow was fore-milked, in line with pre-milking routines to check for mastitis and udder health. This also served to clear any milk that could have a higher number of bacteria than the rest of the milk, again making a cleaner sample. Finally, an equal sample from each teat was taken into a sterile 50 ml tube, taken from the front left teat first then moving to the next in counter-clockwise order to limit contamination (Fig. 2.2). Every person who took samples during the study was taught the correct procedure to minimise contamination risk.

Figure 2.2. Cleaning of teats for milk sample collection

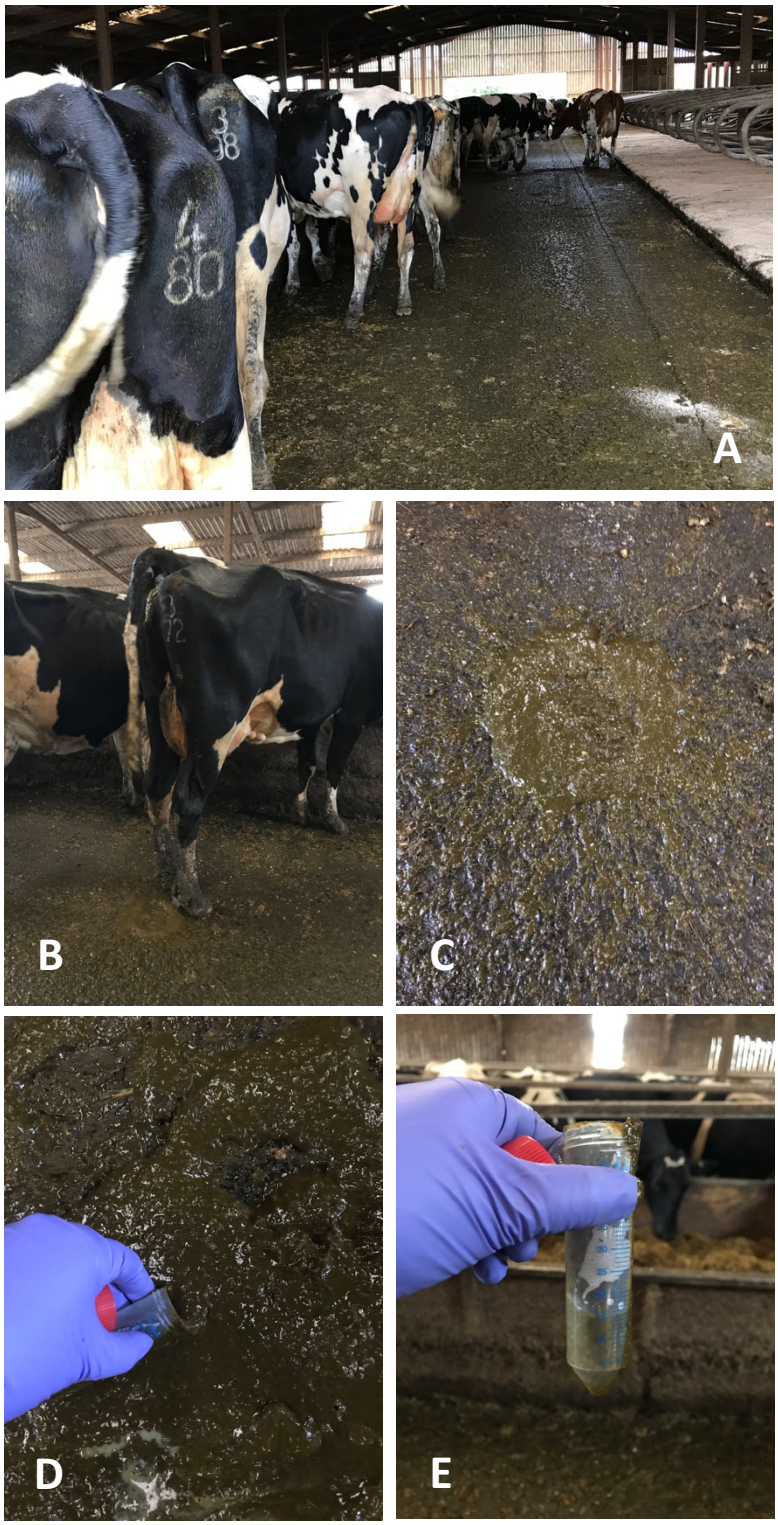


Milk sampling by hand requires a high level of cleanliness to reduce the risk of contamination. Teats were cleaned by the herdsmen as per their normal milking protocol (A), they were then wiped again using ethanol-based wipes by the sampler (B). Fore-milk was discarded (C) and a 50 ml sample taken evenly from all four teats starting from the front left (D)

2.4.3 Faecal samples

Random faecal samples were collected after morning milking and the sheds had been cleaned of slurry. Once entering the shed the cows were allowed a five minute acclimatisation period to allow them to relax and not cause any undue stress. Cows were then observed and a sample collected from the pat when a cow had defecated. Samples were taken from the centre of the pat, so as to not be in contact with slurry (Fig. 2.3). Samples were only taken from suitable pats, where contamination with the surrounding slurry was limited.

Figure 2.3. Collection of faecal samples



Cows were observed in their natural environment after morning milking (A) for defecation (B and C). Samples were then taken immediately from the centre of the pat (D) so to reduce contact with other faeces. Tube were only partially filled (E).

2.5 PHAGE-PCR ASSAY

2.5.1 Milk processing

Samples were collected from the farms during milking using either the sample collection bottles, or by hand milking, as described above. The samples were then transported back to the lab and stored at 5 °C overnight. Whole samples (50 ml) were then centrifuged at 2,500 x g for 15 min to allow the separation of the cream. The cream was then scooped off and the skimmed milk removed. The pellet was then resuspended in 3 ml of M+, and re-centrifuged for 2,500 x g for 10 min as a wash step. As before, the supernatant was aspirated and the pellet resuspended in 1 ml of M+ ready for the phage-PCR assay.

2.5.2 Controls

Control samples were important within the assay to provide confidence that firstly, the reagents were working, and secondly that there is no contamination. A negative control was set up by adding 1 ml M+ into a tube. This control allows confirmation that the bacteriophage was reliably destroyed by the virucide, as there are no mycobacteria in the sample. The positive control was set up by taking 1 ml of a 10^2 cfu ml^{-1} dilution of *M. smegmatis* that has been cultured for use within the assay. Plaques are expected to be formed on this plate, as it confirms that bacteriophage infection occurred.

2.5.3 Assay

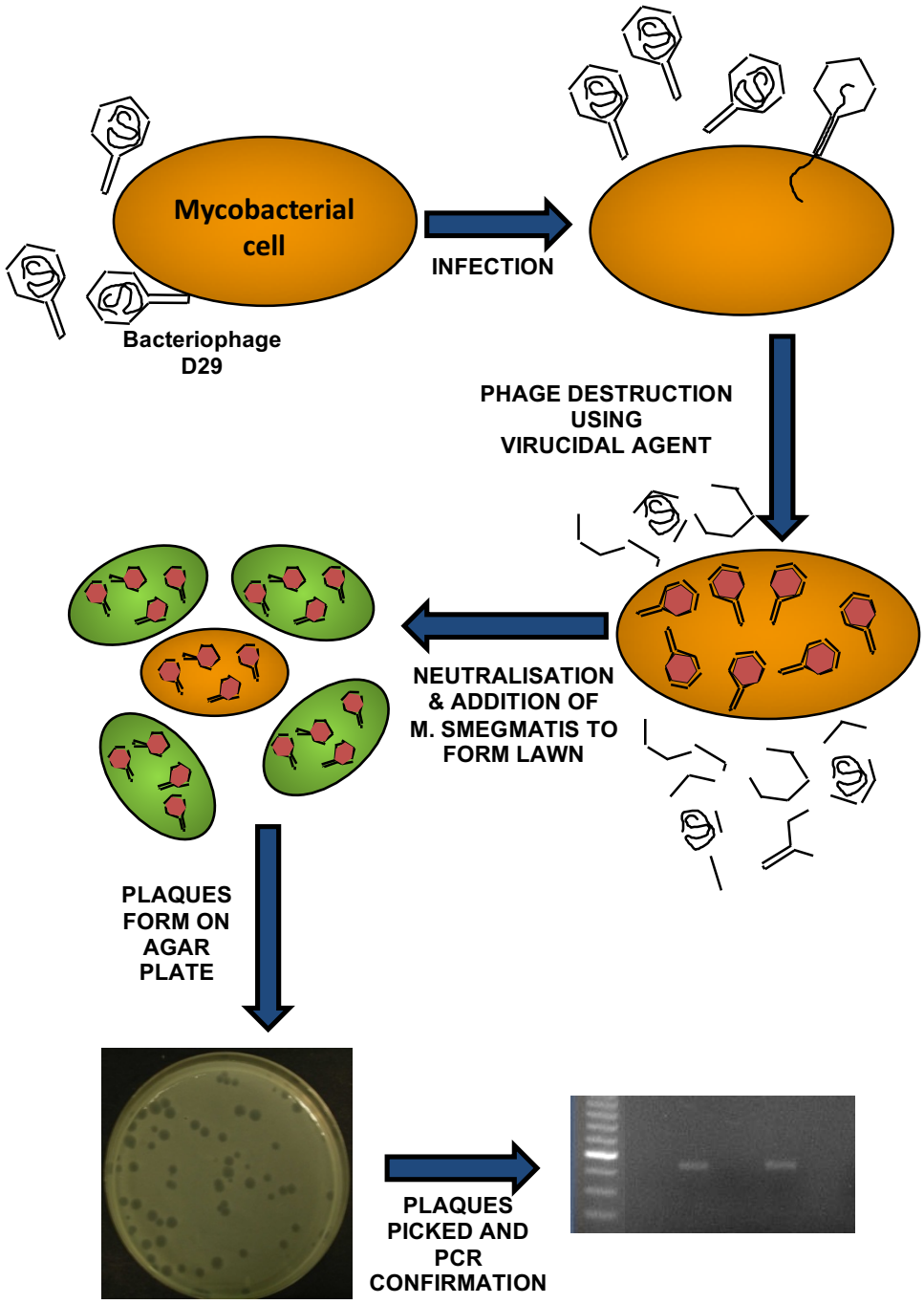
The phage-PCR assay (Fig. 2.4) was carried out as described by Stanley *et al*. (2007), Botsaris *et al*. (2010) and Swift *et al*. (2013). Mycobacteriophage D29 was added (0.1 ml) to the prepared sample and incubated for 1 h at 37 °C to allow phage infection of mycobacterial cells present in the sample. Virucide was added (0.1 ml) and incubated, spinning, at room temperature for 5 min to inactivate any exogenous phage. The virucide was then diluted by addition of 5 ml M+ and fast growing *M. smegmatis* cells added (1 ml) before pour-plating with 7H10 agar (6 ml). Plates were then incubated for 24 h at 37 °C and plaques counted before being picked in groups of five and frozen at -20 °C for DNA extraction.

2.5.4 DNA Extraction

A pooling system of five plaques per extraction was established by Swift *et al*. 2013, and such was followed here. DNA was extracted using the Zymoclean™ Gel Extraction Kit (Zymo Research, USA). Agar from the plaques was dissolved with agarose dissolving buffer for approximately 15 min at 55 °C, then the resulting liquid placed into the spin column within a collection tube. The spin column was centrifuged at 13,000 x g for 30 sec to force the liquid through the silica column to allow DNA to bind and impurities to pass through. DNA wash buffer (200 µl; 96 ml 100 % ethanol and concentrated wash buffer) was added and centrifugation repeated. A second aliquot of wash buffer (200 µl) was added and centrifuged for 1 min at the same speed as above. The spin

column was then removed from the discard tube and placed in an eppendorf tube to collect the DNA. Water (10 µl), that has been pre-heated to 55 °C to give better DNA yield, was added to the column and allowed to incubate for up to 1 min before being centrifuged at 13,000 x g for 1 min. DNA was then frozen at -20 °C until it was needed for PCR confirmation.

Figure 2.4 Summary diagram of the phage-PCR assay



Live mycobacterial cells within the sample are infected with bacteriophage D29. An iron-based virucidal agent destroys all bacteriophage that are not within the protection of a mycobacterial cell. M. smegmatis is added to form a fast growing 'lawn' when plated. Bacteriophage replicate within the original cell, burst open from the original cell and infect those surrounding to form a plaque. These are then 'picked' and presence of MAP confirmed by PCR

2.6 POLYMERASE CHAIN REACTION

2.6.1 IS*900* Nested PCR

The presence of MAP DNA was detected using an IS*900* specific nested PCR, as described by Bull *et al*. (2003) and used by Swift *et al*. (2013). Briefly, a 20 µl reaction volume was used containing 0.2 µM primers TJ1 (5'-GCT GAT CGC CTT GCT CAT-'3) and TJ2 (5'-CGG GAG TTT GGT AGC CAG TA-3'), 6 % DMSO and Hotstartaq Plus master mix (Qiagen; UK). Cycling conditions were as follows; 95 °C for 4 min as an initial denaturation and to activate the *Taq* enzyme, then 10 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 7 min. For the second round, primers TJ3 (5'-CAG CGG CTG CTT TAT ATT CC-3') and TJ4 (5'-GGC ACG GCT CTT GTT GTA GT-3') were used with the following conditions; initial denaturation at 95 °C for 4 min, then 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 7 min. Samples were loaded into a 1 % agarose gel, and ran for 1 h at 80 V.

2.7 CULTURE

All culture work was conducted by SAC Consulting, St Boswells and Edinburgh, using the ESP Trek Diagnostics Systems as detailed below.

2.7.1 Decontamination

2.7.1.1 Milk

Samples are centrifuged for 30 min at 1865 x g and milk fraction discarded, retaining the cream and pellet. These fractions were resuspended in 5 ml of phosphate buffered saline (PBS) and 5 ml of 1.5 % NALC-NaOH (N-acetyl-L-cysteine – sodium hydroxide) and incubated at room temperature for 15 min. Another 15 ml of PBS was then added and centrifuged for a further 30 min at 1865 x g and the supernatant discarded. The cream and pellet were resuspended in 1 ml PBS.

2.7.1.2 Faecal

A faecal sample was added to RO water and allowed to sit for at least 30 minutes to separate. The top segment was removed from the tube and added to 0.9% HPC in half strength BHI broth and incubated overnight in 37° CO_2 incubator. The sample is then centrifuged at 1500 x g for 30 min and the pellet resuspended in the antibiotic brew (para-JEM AS; Trek Diagnostic Systems, US) before incubation overnight under the same conditions.

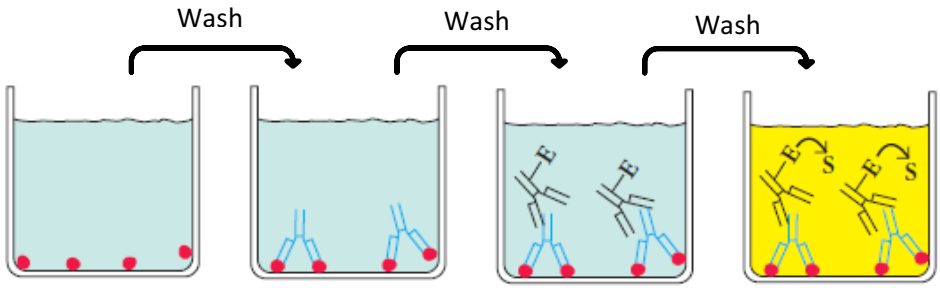
2.7.2 ESP TREK Liquid Culture System

Samples are then loaded into the ESP culture system where they are continuously monitored for growth using a pressure sensor for gas release up to 42 days. If a positive signal is indicated before this point the sample is removed and stained with Ziel-Neelson for microscopic examination and sent for PCR confirmation. If the organisms on the stain are not typical of MAP the sample is either re-decontaminated and inoculated into another bottle or discarded and another sample requested. If, by 42 days, there is no positive signal, the sample is removed and an aliquot used for PCR examination. Samples that have a positive signal are graded as follows, heavy shedder less than 21 days, moderate shedder 22 – 28 days, light shedder 29 – 35 days and very light shedder 36 – 42 days.

2.8 ENZYME-LINKED IMMUNOSORBANT ASSAY

All farms included in the study took part in a monthly recording plan, run by either National Milk Records (NMR) or the Cattle Information Service (CIS), whereby monthly samples were taken to assess the quality of the milk by testing the somatic cell count, butter fat, protein and urea. Every three months an ab-ELISA for JD was also carried out. Both milk recording companies used the same ab-ELISA, IDEXX Paratuberculosis Screening Ab Test (IDEXX Laboratories, US), which is an indirect format that detects the level of MAP-specific antibodies in a variety of matrices including milk, serum or plasma of cattle, sheep and goats. It is quoted as having 60 – 80 % Se and a Sp of >99 % versus culture. Figure 2.5 shows how this assay works up to the point of optical density (OD) readings, where a reading of over 20.00 OD_{600nm} is classed as an inconclusive and over 30.00 OD_{600nm} is a positive result, with an increased amount of antibody in a sample causing an increase in the final OD.

Figure 2.5. Schematic of an indirect ab-ELISA



Wells are antigen-coated, making the antibody within the sample (milk, serum and plasma) attach to the antigen, enzyme-conjugated secondary antigen is added, then finally a substrate that binds to the secondary antigen so that the 'colour' or the level of antibody can be measured.

2.9 DATA ANALYSIS

Data was collated and cleaned in Microsoft Excel 2013 and all analysis carried out in the statistical software programme GenStat (version 16, VSN International, UK). A residual maximum likelihood (REML) model was used for all analysis. This type of analysis was used to estimate the variance of each group, then compare to the other whilst taking into account fixed and random effects. It is deemed to be more appropriate that maximum likelihood models when there are unknown parameters in the model (Speed, 2004), such as the analysis of all animals across farms and lactation and other un-defined groups within one dataset. It is also robust for data that strays slightly away from normal distribution (Smyth & Verbyla, 1996; Speed, 2004). Differences were shown as means (Mean $\pm$ 1.96 x Standard Error). Basic analysis, such a t-tests, were also used to look for differences in the means between two groups of data when no other variables were present.

CHAPTER 3

CROSS SECTIONAL SURVEY OF SIX FARMS WITH JOHNE'S DISEASE USING PHAGE-PCR, AB-ELISA AND MILK CULTURE AS DIAGNOSTIC ASSAYS

3.1 INTRODUCTION

The current JD diagnostics outlined in Chapter 1 have low Se, especially in young cattle. They have a reduced ability to allow farmers to remove animals in the early stage of infection and thus quickly eradicate the disease from a herd. Exposure of young calves often leads to latent infections lasting many years without causing any harm to the cow, however these animals will actively shed MAP into their faeces and milk before showing any clinical signs (Nielsen & Toft, 2008). The main diagnostic used on commercial dairy farms in the UK as a routine JD monitoring tool is an indirect ab-ELISA (IDEXX, US), which is used quarterly on milk samples taken using the sample collection systems in the parlour (see Chapter 2).

The aim of diagnostic tests used to control JD is to detect an infected animal even if it is not showing clinical signs, either indirectly, via an immune response, or directly, by detecting the causative agent (Caldow & Gunn, 2002). The JD milk ab-ELISA is used due to its low cost, ease of obtaining samples and rapidity. It detects the animals immune response to MAP by measuring the level of antibodies in a sample of milk (Stabel *et al.*, 2002). However, the Se of the test is reported to be low (between 21 – 61 %; Nielsen, 2010; Nielsen & Toft, 2008). Performance is also known to be affected by age and stage of lactation of the cow, with cows in their first lactation being two to three times less likely to be ab-ELISA positive, and all cows being more likely to be ab-ELISA positive at the beginning of their lactation (Nielsen *et al*., 2002).

Faecal culture is considered to be the Gold Standard method to detect MAP infection, directly detecting MAP in its primary transmission vector (faeces), so providing an indication as to whether the cow is a risk for spreading disease. However, this method requires chemical decontamination to remove fast growing bacteria, but also causes a decrease in viability of MAP, with the best protocol only recovering a fraction of MAP cells present in the original sample, therefore causing a decrease in Se (Grant & Rowe, 2004). This decrease in viable cells, which ranges from 1 – 4 $\log_{10}$ (Dundee *et al*., 2001), means that only animals shedding a large number of MAP will be identified, as decontamination will reduce the bacterial count below the threshold of detection in animals shedding lower number of the organism. Faecal culture is also slow, taking up to 18 weeks for the formation of colonies on solid media, and although it has a quoted Sp of over 99 % there is a chance that false positives can occur via passive shedding (see Chapter 1).

Even though faecal culture is the Gold Standard, it may also be useful to use milk culture as the faecal-oral route of infection is not the only risk to calves. It also gives an indication of MAP load in raw milk that will be going for human consumption. There is also evidence that MAP can be detected in more milk samples than faeces, with Singh *et al*. (2007) detecting MAP in 96.1 % of milk cultures compared to 84.6 % of faecal cultures, although this was only in a small cohort of 26 suspected infected animals in poor condition. However, this number of milk culture positive animals is unusual, with others culturing MAP from 11.6 % of milk samples from cows who had been identified as asymptomatic faecal culture positive (Sweeney *et al*., 1992).

The above issues with the two most popular detection methods all have a knock-back effect for on-farm management and control, due to being unable to rigorously asses which cows are shedding mycobacteria. Many MAP positive cows are missed due to the Se issues, and so may infect the next generation of heifers to join the herd and put other cows at risk, in turn making JD transmission difficult to halt. This is not the only reason that control practices are not successful, as many farms do not have enough shed space, or money to invest in infrastructure, to enable them to calve and keep JD positive cows separately. Therefore, there is a risk that calves will come into contact with MAP infected faeces and colostrum.

To better understand how to control the disease, improved diagnostics are needed. The phage-PCR assay trialled within this series of studies may overcome the problems observed with current diagnostics, drastically reduce time to detection and should give more detailed results back to the farms. The assay detects the causative agent of the disease, rather than the immune response to the bacteria which can be variable, and doesn't require harsh decontamination procedures which effect viability (Mole *et al*., 2007). This is one of the reasons that it is thought to be more sensitive that the other methods, alongside a low limit of detection (theoretically as low as 1 cfu 50 ml^{-1}). The assay is also very quick, with enumeration results on shedding rate being available to the farms within 24 hours, with PCR confirmation taking a little longer. Due to this, more infected cows should be detected earlier than current diagnostic methods, allowing for improved control and management practises.

The aim of the experiments presented in this chapter was to compare the rapid, low cost phage-PCR assay to milk culture conducted by SAC Consulting and milk ab-ELISA carried out by the farm's milk recording company to determine if the phage-PCR assay can detect more infected animals at an earlier stage. This will expand on previous small-scale experiments, outlined by Stanley *et al*. (2007), Botsaris *et al*. (2010 & 2013) and Swift *et al*. (2013), by conducting whole herd testing across multiple sites.

3.2 METHODS

3.2.1 Farms

A cross sectional study of six UK dairy farms (farms A – F) was conducted between December 2015 and March 2016. Each farm was visited once within two days (+/-) of their quarterly milk ab-ELISA test carried out by either National Milk Records (B, C, D, E and F; NMR) or the Cattle Information Service (A; CIS). The testing schemes ran by these companies consists of samples collected quarterly for milk ab-ELISA testing from every milking animal at the same time as their monthly recording samples were taken. All farms had a minimum history of two years of routine test results, with some having up to six years' worth of results available for the older cows in the herd. Four herds were selected for this study as a convenience sample from a list of farms with ab-ELISA positive cows provided by a local veterinary practice. These farms were located in Derbyshire (three; C, D and F) and Nottinghamshire (one; E). Two further farms were recruited, the first (A) formed part of the longitudinal study used to optimise the assay (see Chapter 5) and the final farm (B) in Buckinghamshire was selected due to personal connections and availability of data. All these herds differed from each other in size, breed and management practices, with four being Holstein-Friesian (C, D, E and F) and two Jersey (A and B) and had a range of 90 to 400 cows in milk. All farms had some JD prevention practices, however no farms employed all recommended procedures, with further details of individual farm management practices given in Chapter 2. All farms admitted they were not as stringent with JD

management as they felt they should be, with all saying that some MAP positive cows were likely to be undetected.

3.2.2 Sample collection

Overall, 574 cows were sampled from across six dairy farms in the UK. Some animals (21) were removed from the study due to inconclusive results with the phage-PCR assay caused by user error during sample processing. This was due to a low titre of *M. smegmatis* which did not form a lawn when added to the phage assay plates. All of these samples were from Farm C.

Two parallel hand-milked samples were collected from 100 systematically selected cattle on Farms A - F during morning milking. Samples were then randomly assigned to be milk cultured or be processed with phage-PCR. Those for milk culture were packed into polystyrene boxes with ice-packs and couriered over-night to SAC Consulting, St Boswell. Milk samples were then cultured using the ESP Trek culture protocol, as described in Chapter 2. Phage-PCR was carried out in the University of Nottingham laboratories.

3.2.3 Preparation and culture of inoculated pasteurised milk samples

A set of milk samples were inoculated with a known amount of MAP determined using the phage-PCR assay. These were sent to SAC Consulting for culture to determine viability of postage and assess the culture method (see Chapter 2).

Multiple MAP strains were grown in liquid culture (7H9 broth) over a period of two months. Enumeration of viable MAP cells was conducted using phage-PCR after de-clumping of the culture by vortexing by glass beads. Once enumerated, the MAP culture was diluted to the desired levels, 10^1, 10^2 and 10^3 cfu ml^{-1}, and 1 ml inoculated into 50 ml of fresh pasteurised milk. Although a slightly different matrix than raw milk due to the removal of a proportion of fat during processing, pasteurised semi-skimmed milk was chosen for this experiment as it was less likely to contain MAP. The pasteurised milk was tested using phage-PCR to confirm that there was no MAP present.

3.2.4 Statistical analysis

In the analysis contained within this chapter, random and fixed effects had to be defined within the REML model. Parity, or lactation number, denotes the number of calves the cow has had and is closely linked to age as the older the cow is, the more likely they are to have had more calves. This variable was used as a substitute for age (i.e. date of birth; DOB) as it is readily available from the farm and milk recording companies. The literature states that the older a cow is the more likely they are to be ab-ELISA positive for JD due to the progression of disease, with the probability of being ab-ELISA positive two to three times lower for cows in their first lactation compared to those in other lactations (Nielsen *et al*., 2002). To confirm that this was true within this cohort and assess whether this should be included as a fixed effect, the

animals most recent ab-ELISA score (OD_{600nm}) were tested across all lactations (1 - 6+). There was found to be a significant difference between lactation (p 0.015, variate: ELISA, fixed: lactation, random: farm), with those in later lactations having a higher ab-ELISA score as seen in Nielsen *et al*. (2002), so this was included as a fixed effect. Farm was included as a random effect as there was a large difference in shedding rates, with D and E having many more phage–PCR negative cows and a lower overall shedding rate than the others included in the studies.

3.3 RESULTS

3.3.1 Evaluation of whether a cow's current ab-ELISA score predicts the detection of MAP in milk

Current 'test and cull' control programs recommend that cattle are culled after three consecutive MAP milk ab-ELISA positive results. These results are seen to demonstrate a breakdown in disease control when a cow is progressing into the clinical stages of infection. However, a debate surrounds the interpretation of the JD milk ab-ELISA test when a cow gives a positive milk ab-ELISA result, but all subsequent tests are negative, or intermittently positive, for the rest of her life. The aim of this analysis was to investigate whether these test results represent false-positive results, or whether there is in fact evidence that these cows are shedding MAP into their milk but, as anecdotal evidence suggests, these animals are able to control the disease at a level that does not trigger the release of antibody into milk at detectable levels.

In this cohort, 106 cows (19.2 %) were phage-PCR positive with a range of 1 - 768 pfu 50 ml^{-1}, indicating that they were shedding MAP into their milk at point of test. Just over half of the samples from the tested population (52 %) produced plaques, ranging between 1 - 544 pfu 50 ml^{-1} indicating presence of viable mycobacteria in the milk samples, but were MAP PCR negative. Almost a third of samples (29 %) produced no plaques, indicating that no viable MAP or other mycobacteria were detected. There was no significant difference between the pfu 50 ml^{-1} of the PCR positive animals and the PCR negative

animals (p 0.357, variate: pfu 50 ml^{-1}, fixed: PCR result*lactation, random: farm). Interestingly, no matter what the mycobacterial count of the sample, whether this was low or high, only one pool of five plaques came up PCR positive for MAP in each sample.

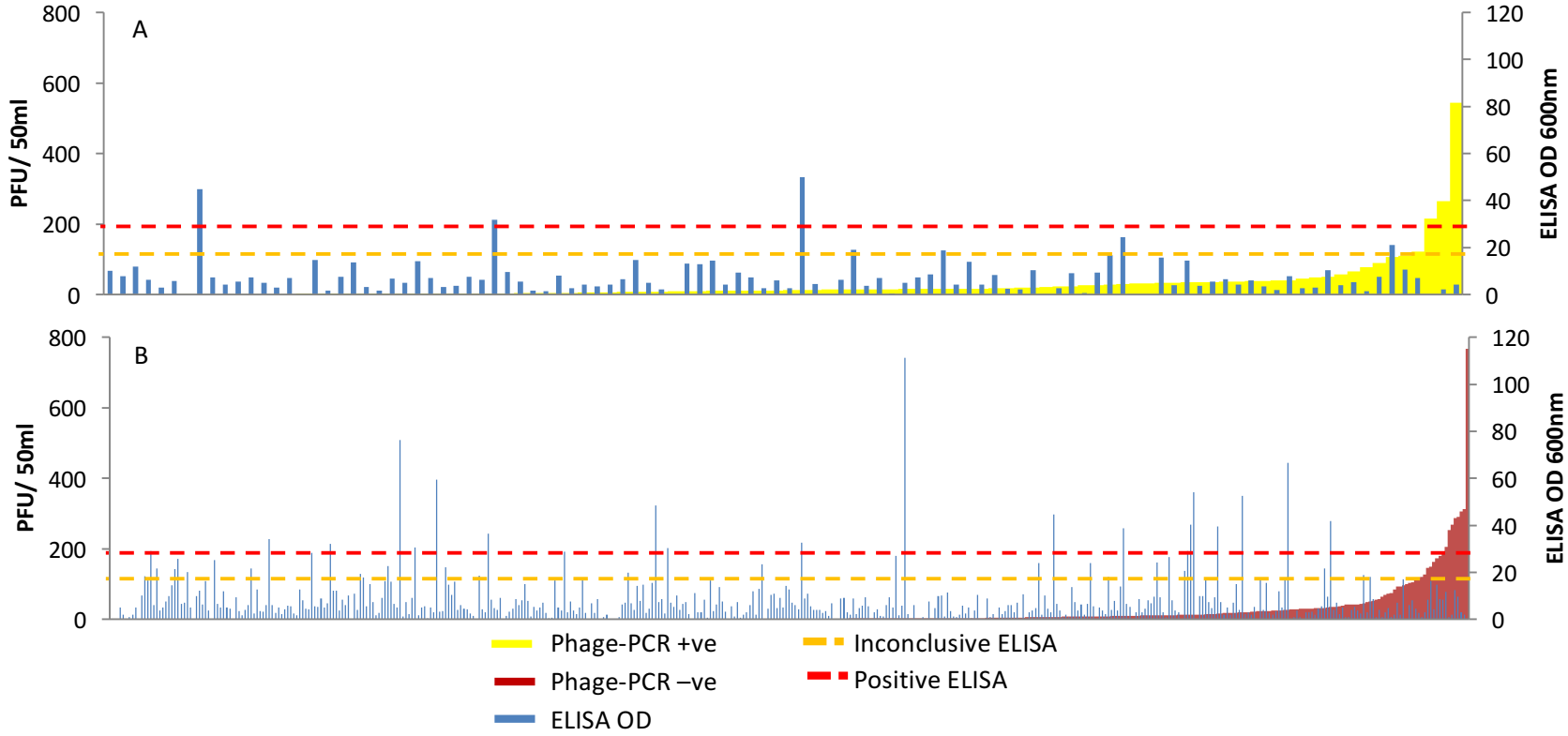
In contrast to these results, only 7.7 % of the cows were MAP milk ab-ELISA positive (> 20 OD_{600nm}) at point of test. ab-ELISA results are split into two categories by commercial testing companies, with OD_{600nm} values > 20 but < 30 categorised as inconclusive results and those giving OD_{600nm} > 30 categorised as MAP-positive. Further analysis of the results gained show that using these boundaries, 3.7 % were positive and 3.9 % gave inconclusive results. However, the results gained here indicate that even a positive milk ab-ELISA test result does not mean that a cow is shedding MAP into milk at detectable levels, with six cows giving a positive milk ab-ELISA result (3.7 % ab-ELISA positive animals) that had no mycobacteria detected in their milk, and a further eleven of those animals that did not give a positive phage-PCR result (6.8 % of ab-ELISA positive animals) being inconclusive, 10.5 % in total.

The results shown in Figure 3.1 visualise the comparison of the individual cow-level from the phage-PCR (pfu 50 ml^{-1}) and their matched ab-ELISA. These graphs show the number of mycobacteria in each sample (pfu 50 ml^{-1}, in order of increasing pfu 50 ml^{-1}) compared to the matched milk ab-ELISA result. There seems to be no link between the milk ab-ELISA result and the number of viable mycobacteria (Fig. 3.1a) or viable MAP cells (Fig. 3.1b) that a cow is shedding in its milk. This means that some phage-PCR negative (viable

mycobacteria present but not confirmed with the MAP-specific PCR) and phage negative (no viable mycobacteria cells) animals are detected as ab-ELISA positive even though they are not shedding MAP into their milk. This also means that many animals who were shedding MAP were negative for MAP-specific antibodies using the ab-ELISA.

Within the phage-PCR results shown in Figure 3.1, there are three easily defined categories; phage negative where no viable mycobacteria were present, phage-PCR negative where viable non-MAP mycobacteria were present and phage-PCR positive where confirmed MAP was present (Fig. 3.1). It would be expected that there would be a difference between the ab-ELISA results for each group as only one of these groups are known to be actively shedding MAP into the milk. No significant difference was found between the current ab-ELISA score of the three groups (p 0.762, variate: ELISA OD_{600nm}, fixed: PCR catagories*lactation, random: farm), suggesting that the milk ab-ELISA result may not be the best indicator of shedding of MAP into milk. In contrast, it is noteworthy that cows with low ab-ELISA results (< 20 OD_{600nm}) which were classed as negative according to the national testing schemes boundaries, were found to be shedding MAP into milk, sometimes at what appears to be very high levels.

Figure 3.1. Comparison of phage-PCR results and ab-ELISA results from all cattle



Only a small number of animals with viable MAP in their milk (phage-PCR positive; yellow) are ab-ELISA (blue) positive (A). Many animal who are have no MAP present in the milk (phage-PCR negative or phage negative) are also ab-ELISA positive (B). There seems to be no correlation between pfu 50 ml $^{-1}$ and ab-ELISA score (results in order of increasing pfu 50 ml^{-1}

3.3.2 Evaluation of whether ab-ELISA history can predict the shedding rate of MAP into milk

The previous section indicated that an animal's current ab-ELISA result may not be the best indicator of shedding of MAP into milk. Therefore, it is possible that ab-ELISA history might provide a better predictor of how the animal is controlling the disease. National testing schemes employed by the farms already use a risk rating system, known as 'J status', which takes into account the last three tests for individual cows. This rating ranges from zero to five, with five being the highest risk level. Definitions for all levels can be found in Table 1.2. A large proportion of the animals within the cohort presented here had a negative MAP milk ab-ELISA history (75 %), which means that they have not had a MAP-positive test result in their testing life (from over two years old). This ranged from two clear tests for first lactation heifers, to up to six years' worth of results for older cows.

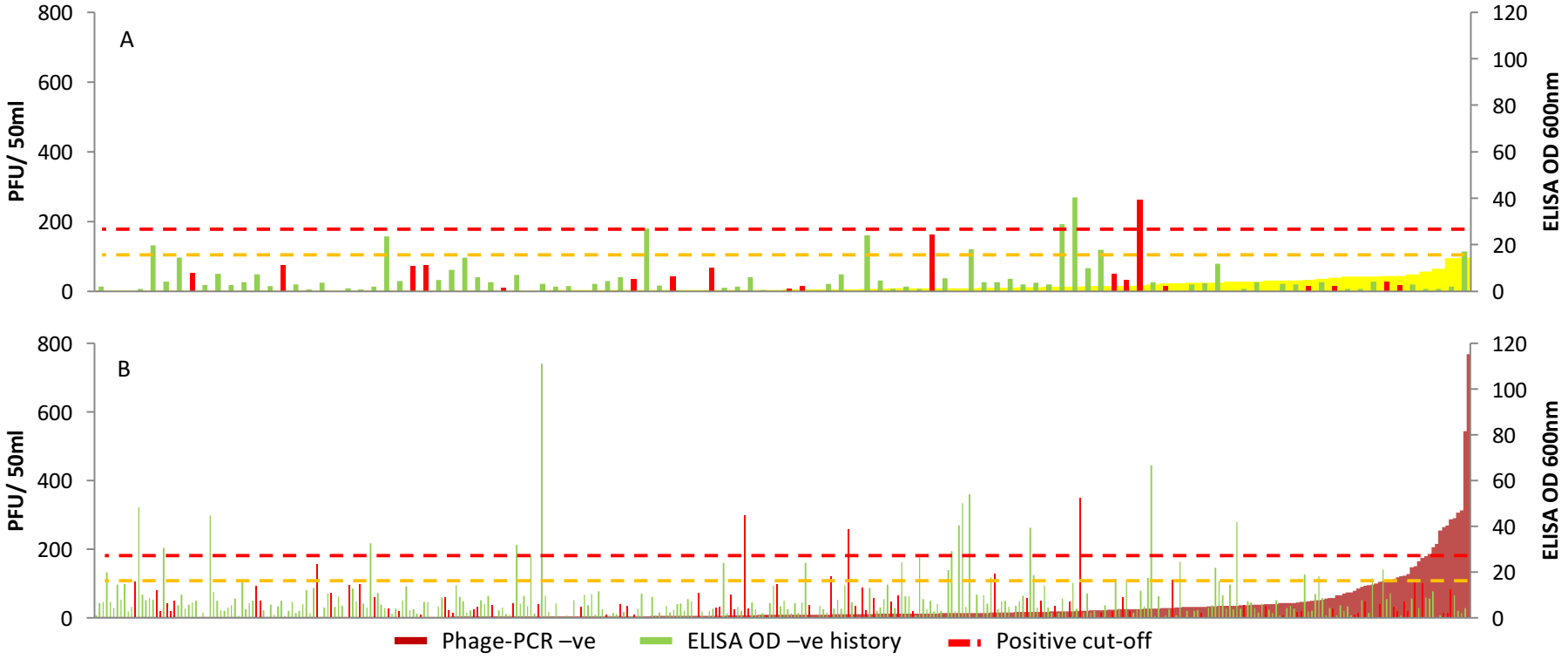
As would be expected, the average ab-ELISA result increases for each J group as assigned by the commercial testing companies. There was no significant difference in the phage results of each J group (p 0.721, variate: pfu 50 ml^{-1}, fixed: J group*lactation, random: farm), any descriptive difference is due to low group numbers. It is worth noting that the 'J status' is a rolling classification so only takes into account the last three tests. This means that there are a number of cows within this cohort classed as J0 who have had an ab-ELISA positive result in the past (14.7 %).

3.3.2.1 ab-ELISA history and shedding rate

The current interpretation of JD milk ab-ELISA test results may not be robust, since this only takes into account the last three tests, meaning that some J0 cows within this cohort have had a positive milk ab-ELISA test result in their history (14.7 %). This is particularly pertinent as the phage-PCR assay results indicated that cows in all classifications were shedding MAP into their milk. To investigate if a simpler classification is more appropriate than the current system, cows falling into two classifications, those with MAP milk ab-ELISA positive history and those without MAP milk ab-ELISA positive history, were compared. This analysis takes into account any positive milk ab-ELISA results that a cow has had, whether these were in the current or any previous lactation. Figure 3.2 shows the comparison of the shedding rate of the cattle with their current ab-ELISA score and ab-ELISA history as defined above. Animals with an ab-ELISA positive history can be found within phage-PCR positive, phage-PCR negative and phage-negative groups. Animals with a high current ab-ELISA positive result do not necessarily have an ab-ELISA positive history. There was no significant difference between the phage results (pfu 50 ml^{-1}) of cows who had a positive ab-ELISA history and those with a negative history (p 0.870, variate: pfu 50 ml^{-1}, fixed: ELISA history*lactation, random: farm). However, Cows with a past positive ab-ELISA also have a higher current ab-ELISA score (mean 15.94 $\pm$ 12.15 OD_{600nm} compared to 6.20 $\pm$ 12.15 OD_{600nm}, p <0.001, variate: ELISA score, fixed: ELISA history*lactation, random: farm)

To take this further, the pfu 50ml^{-1} of the phage-PCR positive and phage-PCR negative animals were analysed separately, therefore taking into account other non-MAP mycobacteria present in the milk. There was no significant difference between the pfu 50 ml^{-1} of the phage-PCR negative cows who have a positive ab-ELISA history and those with a negative history (p 0.872, variate: phage-PCR negative pfu 50 ml^{-1}, fixed: ELISA history*lactation, random: farm). However, there was a significant difference between the pfu 50 ml^{-1} of the phage-PCR positive cows with a positive ab-ELISA history (mean 23.27 $\pm$ 36.88 pfu 50 ml^{-1}) and those with a negative history (mean 67.68 $\pm$ 36.88 pfu 50 ml^{-1}, p 0.037, variate: phage-PCR positive pfu 50 ml^{-1}, fixed: ELISA history*lactation, random: farm).

Figure 3.2. Comparison between phage-PCR result current milk ab-ELISA result and milk ab-ELISA history



Phage-PCR positive animals (A) who had been ab-ELISA positive in the past (red bars) were shedding less MAP (pfu 50 ml^{-1}) than those who had never been ab-ELISA positive (green bars). There was no significant difference between the pfu 50 ml^{-1} of the same groups in the phage-PCR negative group (B)

3.3.2.2 Investigation into the effect of milk ab-ELISA history on MAP shedding rate detected with phage-PCR

The results in the last section showed that animals with a previous MAP milk ab-ELISA positive test result in their history were shedding less MAP than those animals that had a completely negative MAP milk ab-ELISA test history, the next question to be investigated was if there was any influence on this result of the timing of the recent positive result, for example if this positive result needed to be in the previous year, to have this effect. To perform this analysis, three time points were used; less than one year, over one year but less than three years and over three years but less than five years. These time points were chosen other time points resulted in small group sizes. The percentage of animals from the total cohort falling into each of these groups were 17.5 %, 8 % and 2.4 % respectively. A cow could be classified within all of these timeframes if she had multiple positive results within all of the time frames. For example, an animal in her sixth lactation who had an ab-ELISA positive test result each year but negative results in-between would be observed in each of the above timeframes (0.5 % of animals were classified as such).

Within the group of animals who had a MAP-positive phage-PCR, there was a significant difference between the phage-PCR result animals with a ab-ELISA negative history (mean 70.32 $\pm$ 43.75 pfu 50 ml^{-1}), when compared to those who had an ab-ELISA positive test result within the last year (23.98 $\pm$ 43.75

pfu 50 ml^{-1}; p 0.04, variate: phage-PCR positive pfu 50 ml^{-1}, fixed: ELISA positive within last year*lactation, random: farm).

In contrast, there was no significant difference between the pfu 50 ml^{-1} of the phage-PCR positive animals with a positive ab-ELISA history within the two other time frames (> 1 year but < 3 and > 3 but < 5 years) compared to those without (p 0.437 and 0.802 respectively, variate: phage-PCR positive pfu 50 ml^{-1}, fixed: ELISA positive over one year ago*lactation, random: farm).

The difference between ab-ELISA positive and ab-ELISA negative history was not seen in those animals who had a MAP-negative phage-PCR. There was no significant difference between the pfu 50 ml^{-1} in the phage-PCR negative cows who have had an ab-ELISA positive history and those with an ab-ELISA negative history in any of the time points discussed here (p 0.90, 1.00 and 0.128 for < 1 year, > 1 year but < 3 years and > 3 years but < 5 years respectively).

3.3.3 Determining sensitivity of milk culture compared to phage-PCR for detection of MAP

The phage-PCR assay and milk culture can both be used to detect viable bacteria and therefore it was important to compare the Se of these two methods. Botsaris *et al*. (2013) had previously shown that phage-PCR was more sensitive than culture when testing bulk milk samples but individual milk samples are not commonly cultured. The Gold Standard for all JD detection is faecal culture (Donat *et al*., 2017), and a similar method can be used to detect MAP in raw milk samples.

MAP was inoculated into pasteurised milk samples to investigate the survival of MAP in milk samples during postage and to evaluate the limit of detection for the adapted culture methodology.

All samples that had been inoculated with MAP were positive with the ESP Trek culture protocol conducted by SAC Consulting. Samples containing a higher level of MAP K10 were detected by gas signal in the culture machine quicker (15 days) than those with a low level of MAP where no positive gas signal was observed within 42 days. Therefore, these were only detected as MAP-positive with confirmatory PCR after removal from the machine. However, the difference between the high and low MAP-level samples was not seen in the samples containing strains B4 and ATCC, with none showing a gas spike and only being confirmed as MAP-positive with PCR after 42 days (Table 3.1).

The ESP trek culture protocol devised by SAC Consulting has been proven to detect the low levels of MAP observed within raw milk samples, even if this is just by confirmatory PCR after 42 days. This means that it is suitable for use with raw milk samples collected on farm with similar levels of sensitivity as quoted in the literature for faecal culture (see Chapter 1).

Table 3.1. Culture results from pasteurised milk samples spiked with three levels of lab-cultured MAP

MAP Strain	MAP cells per sample (pfu 50 ml^{-1})	Culture Result	Time to Signal (days)[b]
K10	1×10^1	+ve	42 (PCR)
K10	1×10^2	+ve	30
K10	1×10^3	+ve	15
B4	3×10^{1} [(a)]	+ve	42 (PCR)
B4	3×10^{2} [(a)]	+ve	42 (PCR)
B4	3×10^{3} [(a)]	+ve	42 (PCR)
ATCC	1×10^1	+ve	42 (PCR)
ATCC	1×10^2	+ve	42 (PCR)
ATCC	1×10^3	+ve	42 (PCR)

a) 3x rather than 1x is due to a feature of the culture process

b) Samples that do not have a positive signal by 42 days are removed and PCR confirmation carried out

3.3.3.1 The ability of milk culture and phage-PCR to identify animals shedding MAP into the milk

Even though phage-PCR positive animals were found on all farms, milk culture positive animals were only found on three farms (A, D and E). More animals were detected as MAP positive with phage-PCR (19 %) compared to milk culture (2.7 %). Interestingly, there were no animals that were positive on both milk culture and phage-PCR, and six milk culture positive animals had no mycobacteria in the matched phage-PCR assay sample (Table 3.2 and 3.3).

Table 3.2. Comparison between the number of animals detected with phage-PCR and milk culture

	Phage-PCR positive[a]	Phage-PCR negative[b]	Phage negative[c]
Milk culture positive	0	9	6
Milk culture negative	106	277	155

a) plaques and positive with IS*900* PCR
b) plaques but negative with IS*900* PCR
c) no plaques

Table 3.3. Results breakdown of the milk culture positive cows

Farm ID	Cow ID	Phage-PCR		ab-ELISA[a]
		pfu 50 ml^{-1}	PCR	
A	1896	12	-ve	1.00
A	2725	254	-ve	1.00
A	2809	28	-ve	1.00
A	2347	2	-ve	12.00
A	2869	1	-ve	1.00
D	2833	0	N/A	1.14
D	326	0	N/A	3.43
D	3654	0	N/A	0.00
D	51	0	N/A	14.96
D	4031	0	N/A	2.89
D	9404	1	-ve	8.30
D	340	4	-ve	5.96
E	926	0	N/A	2.85
E	871	33	-ve	21.76
E	924	10	-ve	8.56

a) green=ab-ELISA negative; orange=inconclusive ab-ELISA (20 OD_{600nm}); red=ab-ELISA positive (30 OD_{600nm}) as per commercial testing company guidance
N/A PCR not carried out as there were no plaques in phage-PCR

3.4 DISCUSSION

Accurate diagnostics are the key to controlling any disease, with JD being no exception. These are usually judged by the Se and Sp of a test (Whitlock *et al*., 2000); the number of true positives and true negatives as a proportion of the number of animals tested. Current diagnostics for JD are not renowned for having high Se, having a wide range of values depending on the cows age and stage of lactation (Nielsen *et al*., 2002). This results in a large number of cows, sometimes well over 50 %, having a false negative result. McKenna *et al*. (2005) highlights another downfall of the current diagnostics, in that to calculate Se, Sp and other methods to judge the success of a diagnostic, they have to be evaluated against a 'Gold Standard'. In the case of JD, the Gold Standard is culture, whether tissue or faecal, itself a flawed test with low Se estimates. In theory, this test should have 100 % Se and Sp, achieving true detection of all animals. In practice, this is not the case. Results to determine the Se and Sp are interpreted in the context of the history and physical findings, but these are usually out of date estimates. During their evaluation of three different serum ab-ELISA's, McKenna *et al*. (2005) found that Se and Sp were markedly lower when compared to tissue culture (ileum and associated lymph nodes; 8.8 – 16.9 % Se, 90.8 – 97.6% Sp) than faecal culture (13.9 – 27.8 % Se, 90.1 – 97.1 % Sp). Thus they concluded that implementing an ab-ELISA to identify infected cattle will not perform to the expectations set for other infectious diseases where ab-ELISA is used, and the usefulness of the test within a low prevalence herd is questionable. Nielsen (2010) corroborates, adding that temporal aspects of the cow's immune response to

MAP are currently not well understood, so affecting the use and interpretation of immune-test results. This would be better understood if the context of the test is considered, which is where the phage-PCR assay may be able to provide more information.

MAP infections have a long incubation period, sometimes as long as four or five years (Sweeney, 2011), and can result in various outcomes; establishment of infection, shedding in faeces, bacteraemia resulting in shedding in milk and transfer in utero and finally decreased milk yield and weight loss leading to clinical disease (Fecteau & Whitlock, 2010). Stakeholders and decision makers have varying interest in each of these outcomes depending on the setting, for example a herd's prevalence would make a difference in culling decisions made on a low or high shedder, with a low shedder in a high prevalence herd being more likely to be kept than a low shedder in a low prevalence or negative herd (Nielsen, 2010). Immune-based diagnostics compared to culture-based can give a decision maker different information, alerting them to when a cow is first infected, when they are a risk to others by shedding, and when they are no longer controlling the disease. This mixture of testing is currently not used within any control programs, with only the ab-ELISA (milk or serum) being used routinely. However, this may not be an efficient use of these tests, as a cow sheds MAP into faeces for a long period of time before they will become ab-ELISA positive (Nielsen, 2010). A considerable proportion of animals are found to be shedding MAP before two years of age (Weber *et al*., 2010), with Wolf *et al*. (2015) finding 8.1 % of young stock on high prevalence farms shedding MAP when detected by faecal IS*900* PCR.

However, these groups of cattle do not undergo routine testing due to the limits of ab-ELISA (Nielsen & Toft, 2006), therefore they are able to spread bacteria whilst being undetected themselves. Taking this into account, the aim of any new detection method would be to identify infected cows at an earlier stage, so reducing the infectious period by culling at an earlier point, or employing stricter management practices such as isolation of JD positive cattle and snatch calving (others outlined in Chapter 1).

The aim of this series of experiments was to highlight the difference, if any, between phage-PCR, ab-ELISA and milk culture, with each cow having three milk samples taken within one week of each other for each separate test. The phage-PCR assay was conducted within the University of Nottingham's Food Microbiology Laboratory; however, the cultures were conducted at SAC Consulting (St Boswell, Scotland) and the ab-ELISA's by either NMR (farms B, C, D, E and F; Wolverhampton, England) or the CIS (farm A; Telford, England) depending on which company the farm employs for their routine milk recording. As ab-ELISA is the most common detection method used for JD, this was the focus of the investigation. There was found to be no difference between the matched sample ab-ELISA results of those who were phage-PCR positive or negative (p 0.762; Figure 3.1), and a general lack of correlation between the two tests also observed, so those animals who had a high ab-ELISA (immune) response were not necessarily those who were shedding within the milk, and vice versa. Although it would have been good to have two tests that conferred, unlike those currently in use, this finding was not surprising as it is well-known that ab-ELISA produces a proportion of false-

negative results due to the lack of Se and the tests both identifying different elements, or stages of disease. Worryingly, there were several animals who were not shedding any mycobacteria - MAP or otherwise - but had an ab-ELISA score of over 20 OD_{600nm}, so classed as positive and a risk to others. In fact, it could be suggested that this group of animals are fighting disease as they have a sporadic immune response, but are not shedding any live mycobacteria. The phage-PCR assay also highlights the main issue with ab-ELISA, in that it gives no indication of bacterial load or disease status, but rather a snapshot of the immune response of an animal at that point in time.

The literature indicates that there could be serological cross-reactivity with other non-MAP mycobacteria such as *Mycobacterium avium* complex (Stewart *et al*., 2007). The two ab-ELISA's used within the United States have reported specificities of 95.3 and 99.7 % when used to evaluate well characterised samples (Roussel *et al*., 2007). However, when used in the field, the apparent false-positive rate can be much higher than expected, with exposure to some mycobacteria also being shown to cause false results in intradermal tuberculin tests for bTB. A group in Australia isolated nine strains of atypical mycobacteria and one *Rhodococcus* from soil samples, then injected a number of animals subcutaneously (Corner & Pearson, 1979). Each animal was then tested with bovine and avian tuberculin four and ten weeks after infection. Six of the strains showed a significant level of Se to both tuberculin's at four weeks, but only one of these went on to be detected at ten weeks. This shows that there can be some cross-reactivity between mycobacteria strains. A similar study conducted by Roussel *et al*. (2007)

concurred with Corner & Pearson (1979), after concluding that there can be an apparently high rate of MAP infection by ab-ELISA among certain groups of animals, despite the animals never developing clinical disease and a lack of isolated MAP organisms. However, it was noted that non-MAP mycobacteria were isolated from faecal samples collected from these herds. They found that cattle from six affected herds were 9.4 times more likely to have environmental mycobacteria, such as *Mycobacterium terrae* and *Mycobacterium avium* complex, isolated from faeces than three geographically matched comparison herds. Another experimental trial was conducted by Osterstock *et al*. (2007) on nineteen weaned crossbred beef calves infected with a number of different mycobacterial isolates including *M. avium*, *M. intracellulare*, *M. scrofulaceum* and *M. terrae*. This caused cross-reaction (false positives) in both ab-ELISAs used in the study, leading the group to conclude that environmental mycobacterium may represent a high proportion of false-positives serologic reactions observed in the field.

Alternatively, false-positives could occur through an innate immune response. Olsen & Storset (2001) investigated this theory by using eight orally infected (10 mg/ml wet weight bacteria each day for ten days from three weeks of age), and eight uninfected controls housed separately. All calves were sourced from a herd with no history of JD and a current whole herd negative test with ab-ELISA. Blood samples were taken from animals at multiple time points and tested for an IFN-γ response to two MAP antigen proteins, MPP14 and PPDp. The response measured in the uninfected groups, who were housed separately to the infected animals, gradually declined from the age of three

weeks with next to no response seen after 5 months. However, two calves from this group responded repeatedly, but not consistently to both antigens. Additionally, five calves from another herd with no JD history were tested. All calves responded to the INF-γ test, four to MPP14 and one to PPDp. This demonstrates that there is a high degree of innate response to antigens from mycobacteria. In another study, 28 cows from farms that used 'test and cull' management for JD were purchased and calved from mid-winter to early summer. Calves were then split into four treatment groups; naturally exposed, artificially infected, vaccinated, and controls. They, like Olsen & Storset (2001), found that all calves had a INF-γ response at least once throughout the trial, including the control animals. In addition to the JD INF-γ response, McDonald *et al*. (1999) explain that seven of the calves also had a response to bovine tuberculosis, even though this has been eradicated in Australia, where the study was conducted. Jungersen *et al*. (2002) adds that until novel antigens that are more specific for the diseases are available, the interpretations of immune-based tests need to be individually adjusted to fit specific needs and the context of which the test is used. However, this does not seem to be taken into account when using the ab-ELISA in the field, with repeat testing to eliminate the risk of culling a false positive being taken into account. The studies outlined above could provide an insight as to why there are a number of cows in the current study who have an immune response detected with ab-ELISA, but are either not shedding or have non-MAP mycobacteria in the milk. This again highlights the issues with the use of an ab-ELISA as a routine test to provide information on the disease status of an

animal, and gives more evidence as to why the phage-PCR assay has potential to be used to give a more detailed picture of the disease status and shedding rate of a herd.

The quarterly JD testing regimes run by commercial testing companies provide a 'Johne's risk' status for each cow tested. This allows the farm to make more informed decisions of the risk a cow poses to others, as a cow with more positive tests is assumed to be a higher risk to those that have just have one positive test (see Table 1.2 for an example of the rankings). The phage-PCR assay measures the number of mycobacteria in the milk so it is interesting to compare this to ab-ELISA history, as an animal's current ab-ELISA score is not linked to the number of mycobacteria in the milk. However, this study has shown that a cow who has had a positive ab-ELISA result, so has exhibited an immune response, in the previous year is shedding less MAP (23.98 $\pm$ 43.75 pfu 50 ml^{-1}) than one who has never had a positive ab-ELISA (mean 70.32 $\pm$ 43.75 pfu 50 ml^{-1}). This is an interesting finding as it is opposing the knowledge given to farmers, where any positive is a risk to others as it will have had to have been exposed to MAP to elicit an immune response. Which although still true, these cows may also be controlling the disease compared to those who have not had a positive ab-ELISA, therefore no or limited immune response. Cows with a past positive ab-ELISA also have a higher current ab-ELISA score (mean 15.94 $\pm$ 12.15 OD_{600nm} compared to 6.20 $\pm$ 12.15 OD_{600nm}), meaning that they have the specific antibodies in circulation to be able to deal with a MAP breakdown as and when it happens. The

findings that animals who have had a positive ab-ELISA in the last year shed less MAP and have a higher current ab-ELISA score combined could suggest that a single positive may give a 'protective effect' in the short term. This could mean that once the immune system has responded to infection, there could be a clearance of MAP and an increased amount of specific antibodies in circulation. However, this 'protective effect' does not seem to apply if the ab-ELISA positive was over a year previous, with no significant difference between the shedding rate of animals who have had a positive over one year but within three years and over three years but within five years and those with a completely negative history. This finding could be compounded by the average lifespan of a dairy cow, which is two to three lactations, so the number of animals in this analysis were small (44 and 13 respectively).

The current advice given to farmers is to breed any cow who has had a positive ab-ELISA, be that single or multiple, to a terminal sire (e.g. a dairy animal will be inseminated with semen from a beef bull). This means that offspring will be reared and enter the food chain rather than being used as replacement heifers, so reducing the number of infected animals entering the herd. Upon review, the findings reported here should not change the advice given to farmers. Intermittently ab-ELISA positive animals have still been exposed to MAP as they have antigens in circulation, and are at risk of disease breakdown. To understand this further, more work should be undertaken to review what is known - and unravel some of the unknowns - with regards to genetic susceptibility as the phage-PCR assay provides better detection of MAP-shedding and is easy, quick and cheap for research purposes. Kirkpatrick

& Shook (2011) suggest that selective breeding is a good candidate for preventing infection. However, genetic improvement of a herd is a slow, long-term process. Results are permanent as any gains will benefit future generations. As has been shown in research discussed in this chapter, the phage-PCR assay can be used alongside ab-ELISA to identify different groups of animals; infected, infectious and recovered. The identification of infection status means that the genetics of the different groups can be studied and those whom have recovered can be bred from to reduce the disease susceptibility of the herd. The use of phage-PCR would reduce the reliance on ab-ELISA to make breeding decisions. The results from the phage-PCR also highlight that the industry could have been culling the wrong group of animal's due to current reliance on ab-ELISA testing.

Parallel milk samples were also taken for culture at SAC Consulting (St Boswell), as this is a comparable culture-based method to phage-PCR. However, this is not yet a validated test as it uses a different sample matrix than the Gold Standard of faecal culture. Limited validation for this study was carried out. Three concentrations of three strains of MAP (approximately 10^1, 10^2 and 10^3 cfu 50 ml^{-1} of K10, ATCC and B4) were sent on ice via overnight courier to SAC Consulting to monitor the effects of transport and check that the decontamination procedures were successful. All levels of MAP, including the lowest levels, were detected by culture method, which was surprising considering the evidence regarding loss of viability (Bradner *et al*., 2013; Dundee *et al*., 2001; Grant & Rowe, 2004). The results from the first set of samples (K10) were as expected, where the 10^3 cfu 50 ml^{-1} were detected by

the change in pressure from the release of gas during growth much quicker (15 days compared to 30 days) than the 10^2 cfu 50 ml^{-1} samples. The final concentration (10^1 cfu 50 ml^{-1}) could only be detected with PCR after completing the full 42-day cycle in the machine, meaning that the MAP cells contained within the sample were in such low numbers that they could not be detected. Interestingly, the other strains of MAP were only detected via PCR confirmation of negatives, much like all of the positives obtained from the clinical samples.

A number of the milk culture positive samples were phage-PCR negative (40 %). The lack of correlation between the two culture-based methods could have been caused by the sample collection method, where the two samples were collected one after another then randomly assigned to culture or phage-PCR. If this type of sample collection were to be repeated it would be better to collect one large sample, for example 100 ml, mix and split into two samples before completing the two separate tests. However, after further investigation even this may not change the results seen here due to MAP possibly being held unevenly within the somatic cells (Chapters 5 and 6).

In conclusion, the phage-PCR assay potentially provides a more in-depth picture of what is happening during infection, providing a shedding rate rather than positive or negative as in the case of culture or PCR. It could provide quicker detection due to looking for the growth and replication of bacteriophage within the MAP cell, rather than the extremely slow growth of the bacterium. Alongside this, it provides live-dead differentiation as the

phage will only infect a live cell (Botsaris *et al*., 2013; Stanley *et al*., 2007; Swift *et al*., 2013). This lowers the risk of false-positives, as a cow who is fighting the disease so has an increased immune response and the DNA of dead MAP within the sample matrix will not be detected. One of the main reasons for using ab-ELISA on-farm rather than other available tests is cost, with commercial testing companies charging £0.50 to £0.78 a month per cow for a quarterly testing programme, depending on the number of animals in the herd (1000+ down to 50 respectively; NML, 2011). Compared to culture, where farms are charged approximately £42 per sample (SAC Consulting, 2016), ab-ELISA is much more cost-effective. Phage-PCR can combine the cost and speed of ab-ELISA, but provides detection of the causative agent of the disease. This will give the decision maker more information on an animal's risk of infecting its offspring. However, it may not be in the best interest of disease control to abandon ab-ELISA and replace the current testing scheme or culture with phage-PCR, as ab-ELISA is currently the best detection method on the market. It would be more useful to use it alongside these other tests to provide more in-depth information on the progression of disease.

CHAPTER 4

A CROSS SECTIONAL SURVEY OF DAIRY CATTLE ON SIX FARMS USING PHAGE-PCR, MILK AND FAECAL CULTURE AS DIAGNOSTIC ASSAYS

4.1 INTRODUCTION

Even though ab-ELISAs are widely used for control programs due to their speed and low-cost, as discussed in Chapter 1, they are not the Gold Standard for JD detection. Culture methods, specifically faecal, are used to gain an indication of the amount of MAP that a cow is shedding and therefore determine the risk that they pose to others. However the long incubation times (7 weeks in broth and 16 weeks on solid media), costs of approximately £42 per sample (SAC consulting, 2016), and intermittent shedding of MAP hinders the efficient use of this method in screening programs (Laurin *et al.*, 2015). As described in Chapter 1, faecal culture is carried out using both solid and liquid medium, with liquid being the more common for commercial detection. The main limitation that affects all culture methods is decontamination of samples to remove fast growing bacteria. This can also affect the viability of MAP, especially when the sample contains low levels.

Faecal-oral transmission is accepted as the primary dam-daughter transmission route (Sweeney, 1996). Adult cattle in the clinical stage of disease can shed 10^6 to 10^8 cfu g^{-1} MAP in faeces (Jørgensen, 1982). A calf can become infected by ingesting levels as low as 50 to 10^3 cfu (Chiodini, 1996), so it is prudent to remove animals that are shedding MAP in faeces from the herd as quickly as possible. Although faecal culture is not used widely within control programs in the UK, it is in countries such as the USA and the Netherlands where annual testing of individual animals is carried out. In the UK, it is used on an *ad hoc* basis, either to confirm infection in a small group of

animals or using a pool of samples from multiple animals to reduce the cost of testing. Faecal culture has also been used in conjunction with environmental sampling of cattle-housing to establish the herd-level shedding rate by collection of slurry and effluent from alleyway floors (Berghaus *et al*., 2006; Donat *et al*., 2017; Lombard *et al*., 2006).

Even though faecal culture is not widely used within UK control programmes, it has been used in comparative studies to determine the performance of other tests. Stabel *et al*. (2002) attempted to estimate the percentage of seropositive herds with cows shedding MAP into the milk and faeces. They found that of the 24 herds that had at least three cows who were ab-ELISA positive, 79 % also had cows who were culture positive for MAP. However, only 25 % of the cows who were shedding MAP in their milk or faeces were also ab-ELISA positive indicating that there is not a direct correlation between ab-ELISA status and shedding of bacterial into faeces. Contrary to this, Beaver *et al*. (2017) identified a strong association between milk ab-ELISA and faecal culture in repeated faecal sampling of fourteen infected cows from two low-prevalence herds. They employed a statistical model that could accurately account for multiple diagnostic results whilst adjusting for the effects of individual animals and herds over time. They suggested that this approach can provide a more detailed understanding of the interplay between diagnostic outcomes but also acknowledged that the relationship between faecal shedding and milk ab-ELISA status was not simple. Many other authors have used culture to validate other diagnostic methods, and these studies highlight the disconcordance between the different diagnostic tests, and therefore it is

clear that another culture-based method is required to accurately assess shedding rates.

The Se and Sp of new diagnostic tests is assessed by comparison of the results gained to a Gold Standard method. For JD this is problematic as the Gold Standard (faecal culture) does not identify all positive animals, and there are variations in the limit of detection between different culture methods (Eamens *et al*., 2000). However, new statistical techniques have emerged to allow the evaluation of test performance without reference to a Gold Standard (Wang *et al*., 2011). Espejo *et al*. (2015) used this technique to estimate the probability that faecal culture correctly identified three different categories of infected animals. They found that 70.9, 32.0 and 98.5 % of heavy, light and non-shedders were correctly identified. This confirms what is seen in practice, with light shedders often being missed due to culture having a low detection limit due to decontamination procedures.

The phage-PCR assay is comparable to culture methods as it also detects live bacteria. The previous survey established how the phage-PCR assay may perform against milk ab-ELISA when used on a large scale, with milk culture also used to provide a second assessment of MAP shedding into the milk. Therefore, the aim of this follow-up cross-sectional survey was to compare the performance of the phage-PCR assay to faecal culture. This was conducted to determine whether phage-PCR could be used as a good predictor of faecal shedding and could be used within control programs to reduce MAP transmission from the primary source of infection. These two detection

methods will also be analysed in the context of the animal's most recent ab-ELISA score and milk culture, the former because it is used to inform management and control programs within the UK and the latter as it again, provides a second assessment of an animal's shedding rate, albeit into milk rather than into faeces.

This cross-sectional survey will determine if the phage-PCR assay could be used on-farm to detect any animals shedding MAP earlier than other diagnostics, with removal of these animals leading to a quicker reduction in herd-level disease burden.

4.2 METHODS

A number of animals (161) from Farms A (24), B (24), C (25), D (29), E (27) and F (32) were selected to under-go further testing with matched faecal culture, milk culture and phage-PCR tests. The animals were selected on the ability to collect faecal samples as described below, and subsequent milk samples were collected at the next milking. Each herd was observed in their natural environment after the yards had been cleaned of slurry until an animal excreted that could be marked and their number recorded. The placement of the faeces was also observed before the cow was selected to ensure it was not contaminated with faeces from another cow or the surrounding slurry. A sample was then collected as described in Chapter 2. Animals were observed for defecation until the desired number of samples were obtained. Although it is a not a sterile sample, this was deemed more acceptable than taking a sample during routine veterinary fertility visits as most animals presented during these visits will most likely be within a similar lactation stage, so could have caused a selection bias within the results. Two parallel milk samples were then taken in the parlour from the selected animals during their next milking after faeces collection (afternoon for five farms, lunchtime for one). These animals were of varying ages (lactation 1 – 9) and stages of lactation, however there was a natural bias towards younger animals as would be expected in an average dairy herd. The faecal sample and one milk sample was sent to SAC Consulting for culture (Chapter 2), and the phage-PCR assay performed on the second milk sample. These samples were taken within one-

month of the survey described in Chapter 3 so that the milk ab-ELISA results gained in that study were still valid for comparison.

REML analysis was used to assess if any significant relationships existed between the detection methods as described in Chapter 2. As in Chapter 3, farm and lactation were defined as random and fixed effects respectively, as MAP shedding rates differed between younger and older cows and between farms.

4.3 RESULTS

Overall 11.2 % of animals tested in this survey (18/161) were faecal culture positive and these were only found on Farms A (n=9), B (n=3) and E (n=6). As in the previous survey, milk culture was conducted on matched phage-PCR samples to give a second indication of MAP shedding into the milk. Less animals were milk culture positive than faecal culture positive, with only 5.0 % of animals (8/161) being found to be positive. Milk culture positive animals were also only found on Farms A, B and E. Only one animal was positive with both milk and faecal culture methods, found on Farm A.

However, no samples (milk or faecal) resulted in a gas signal in the ESP Trek machine within the 42-day time-period, and so all culture positive samples were detected by PCR after they had been removed from the machine (see Chapter 2). This suggests that only low levels of MAP were present in any of these samples, as higher levels are expected to produce a gas pressure signal before this time point, as seen when the milk cultures were inoculated with known levels of MAP in Chapter 3.

As would be expected, when taking into account the previous comparisons with milk culture in Chapter 3, more animals were phage-PCR positive than milk or faecal culture positive, with 24.2 % (39/161) being phage-PCR positive for MAP, 65.2 % (105/161) being phage-PCR negative (detection of non-MAP mycobacteria), and 10.6 % (17/161) were phage negative (no mycobacteria detected). Again, as seen in the previous cross-sectional survey, there were large differences in the shedding rate between the six farms, with Farms B, D

and F having a lower overall shedding profile than the others. This means that although the presence of non-MAP mycobacteria complicates detection of MAP individually, the phage-PCR assay has the ability to give an indication of whether overall herd-level shedding is high, and whether the disease in endemic or only a small number of cows are infected.

As this survey was conducted within one month of the previous survey (Chapter 3), the previous ab-ELISA results were still valid. This means that they can be used for comparisons to the other assays presented here. Of the animals included in this survey, only eight were milk ab-ELISA positive (5 %; > 20 OD_{600nm}), with 17.4 % of the total cohort of the previous chapter (553) having an ab-ELISA positive history. Further details of on-farm differences are shown in Table 4.1.

Table 4.1. Summary of the pattern of results on the six farms

	Phage-PCR positive[a]	Phage-PCR negative[b]	Phage-negative[c]	Milk Culture positive	Faecal Culture positive	Milk ab-ELISA positive[d] (>20 OD_{600nm})
Farm A n=24	25 %	70.8 %	4.2 %	12.5 %	37.5 %	0 %
Farm B n=24	20.8 %	54.2 %	25 %	12.5 %	12.5 %	20.8 %
Farm C n=25	32 %	68 %	0 %	0 %	0 %	4 %
Farm D n=29	13.8 %	75.9 %	10.3 %	0 %	0 %	0 %
Farm E n=27	29.6 %	63 %	7.4 %	7.4 %	22.3 %	0 %
Farm F n=32	25 %	59.4 %	15.6 %	0 %	0 %	6.3 %

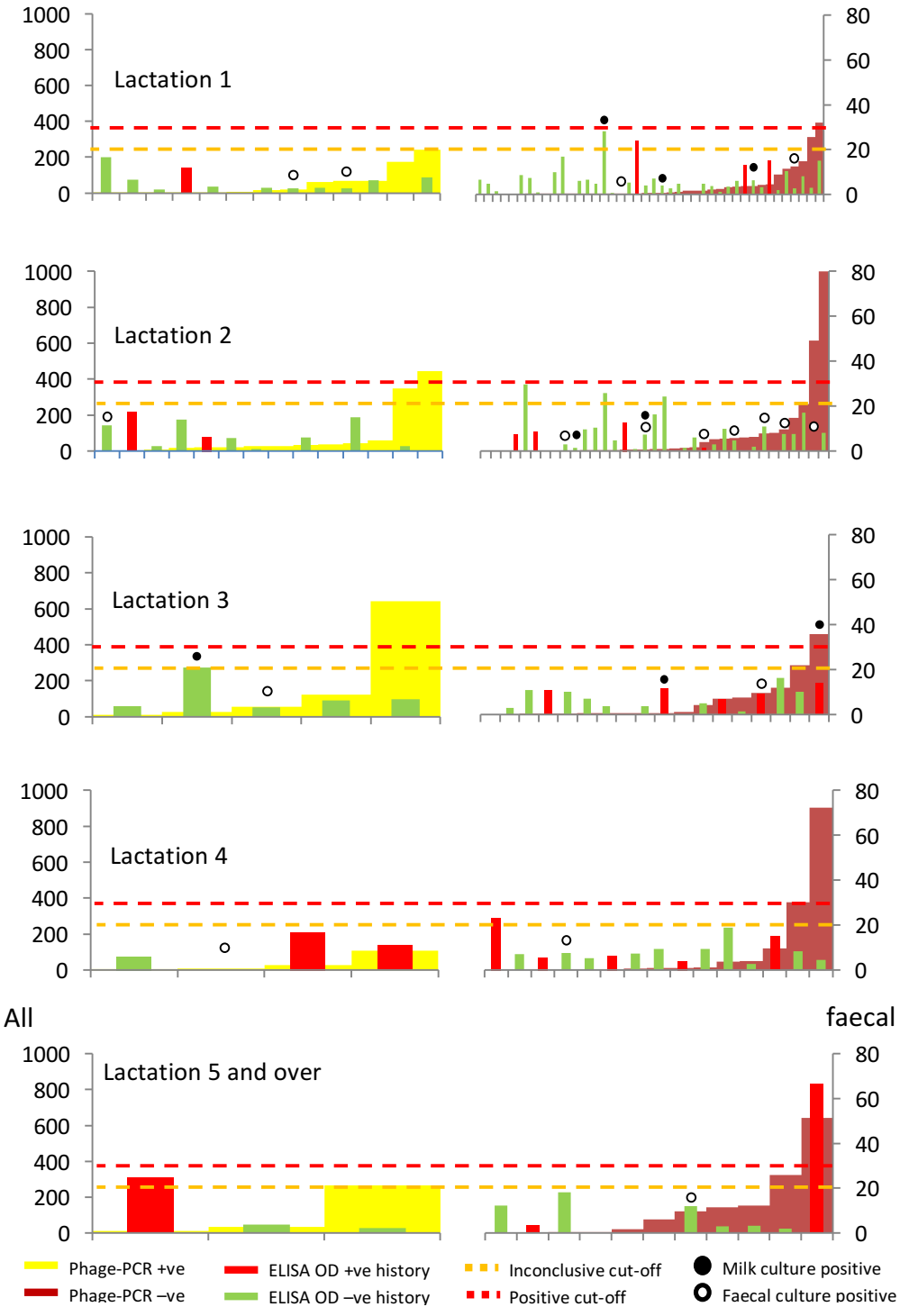
a) MAP positive
b) Mycobacteria positive, MAP-negative
c) No mycobacteria detected
d) Results from milk ab-ELISA analysis in Chapter 3

4.3.1 Comparison of phage-PCR to the current Gold Standard methods of detection

Even though faecal culture is the Gold Standard detection method for JD, it is not widely used. Even though the Se is low, it provides information on the accepted primary route of transmission (faecal-oral), and allows an assessment of whether a potential dam will be a risk to her daughter and other calves. Therefore, it is prudent to assess how the phage-PCR assay compares to faecal culture, and investigate whether it is a good predictor of faecal shedding. It also makes sense to compare phage-PCR to other similar culture-based detection methods to determine if it is more sensitive.

There was very limited cross-over between the three detection methods used here (Fig. 4.1). Only 12.8 % of phage-PCR positive animals were also faecal culture positive (5/39), with no statistically significant difference found between the phage results (pfu) of those who were faecal culture positive and faecal culture negative (p 0.874, variate: pfu 50 ml^{-1}, fixed: faceal culture*lactation, random: farm). However, the lack of statistical significance may be due to the small number of faecal-positive animals within the study. The disconcordance observed between faecal and milk shedding of MAP when comparing faecal culture and phage-PCR was also seen with milk culture as only one animal was positive with both faecal- and milk- culture methods (Tables 4.2 and 4.3). A venn diagram was also constructed of the three culture-based tests, faecal, milk and phage-PCR to observe the agreement between the tests in detection of MAP-positive animals (Fig. 4.2).

Figure 4.1. Comparison of phage-PCR, faecal and milk cultures with ab-ELISA



There is disconcordance between all detection methods presented here, with only one animal having a positive milk (solid circle) and faecal (white circle) culture. A limited number of animals were phage-PCR positive and culture positive (left-hand graphs). Only one faecal culture positive animal had an ab-ELISA positive history (bright red bar)

Table 4.2. Comparison between the number of animals detected with phage-PCR and faecal culture

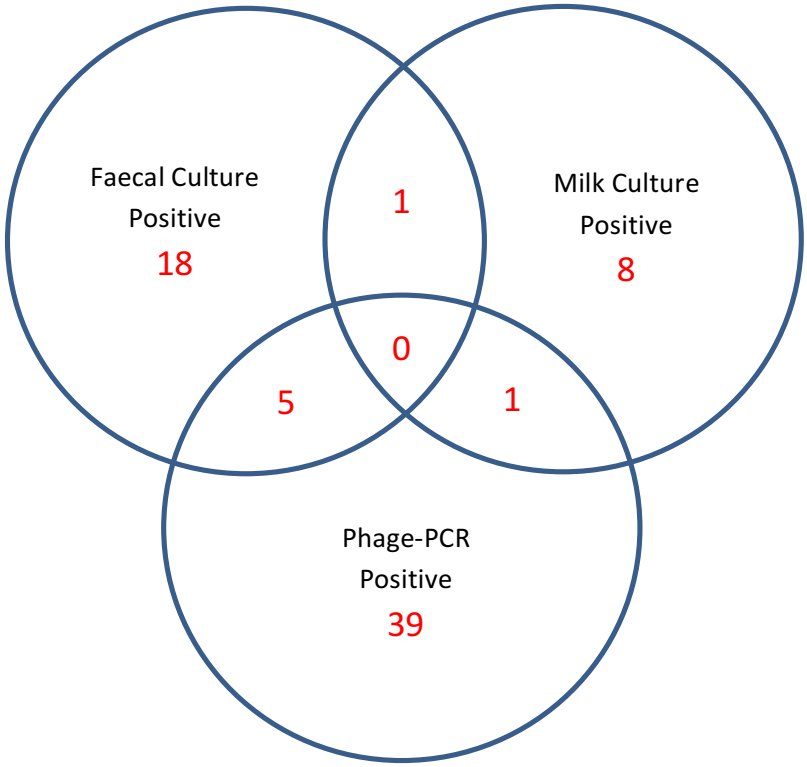
	Phage-PCR positive[a]	Phage-PCR negative[b]	Phage negative[c]
Faecal culture positive	5	11	2
Faecal culture negative	34	94	15

a) plaques and positive with IS*900* PCR
b) plaques but negative with IS*900* PCR
c) no plaques

Table 4.3. Comparison between the number of animals detected with faecal culture and milk culture

	Milk culture positive	Milk culture negative
Faecal culture positive	1	17
Faecal culture negative	7	136

Figure 4.2. Cross over between the positive-results in three culture-based assays



The venn diagram shows the lack of agreement between the three culture-based assays used within this survey, with phage-PCR detecting more MAP-shedding animals than faecal and milk culture.

4.3.2 Comparison of phage-PCR, faecal culture and ab-ELISA

If a traditional faecal shedding curve is considered - whereby an animals shedding rate increases steadily over time - a cow frequently does not give a consistent ab-ELISA response until quite late in the disease progression and it shedding high-levels of MAP in the faeces. It is therefore not surprising that faecal culture positive cows were negative on their current ab-ELISA test, as all samples did not exhibit a gas signal during culture, so were confirmed by PCR, suggesting low-levels of MAP present. There was no significant difference between the most recent milk ab-ELISA score of the faecal culture positive and faecal culture negative animals (p 0.954, variate: ELISA OD_{600nm}, fixed: faecal culture*lactation, random: farm). Only one faecal culture positive animal had an ab-ELISA positive history.

Interestingly, 28 % of faecal culture positive animals (5/18) were found to be also be MAP-positive by phage-PCR before they had had an ab-ELISA positive result (animals were currently ab-ELISA negative and had an ab-ELISA negative history). This represents a group of animals who have been detected as infectious earlier than if milk ab-ELISA was used alone. The identification of animals shedding MAP into the faeces by phage-PCR is likely to be an under-estimation as more of the faecal culture positive animals who were phage-PCR negative may have been MAP-positive but missed due to the plaque pooling protocol whereby three replicates of five plaques were picked randomly from the plate.

Due to samples also containing non-MAP mycobacteria within the milk, 85 % of the phage-PCR MAP positive samples only had one positive replicate. The effect of non-MAP mycobacteria on the detection of MAP is discussed in Chapter 5. The suggested earlier identification of faecal positive animals provided by phage-PCR compared to ab-ELISA could have a significant effect on the control and management of disease.

Thirty-four animals were phage-PCR positive, but faecal culture negative. This may represent an early detection of disease, but it is more likely that it is a feature of intermittent shedding in the early stages of disease.

4.4 DISCUSSION

In this cohort of animals, 13 % were faecal culture positive, which is a high prevalence compared to that reported in other published surveys. Douarre *et al*. (2010) found that 7.9 % of animals were faecal culture positive when strategically selected from across seven herds in Ireland with a history of, or likely exposure to, MAP. However, this could be due to the differences between the culture methods as Douarre *et al*. (2010) used culture on solid media, whereas in this study ESP *para*-JEM liquid culture was used. Williams-Bouyer *et al*. (2000) compared three different methods of culture for the growth and detection of mycobacteria, namely BACTEC MGIT 960 (liquid culture), ESP Culture System II (liquid culture) and Middlebrook 7H11 selective agar. A total of 3,151 specimens of all types (sputum, urine, faecal and tissue) were cultured, with only 7.3 % yielding mycobacteria, the most common being *Mycobacterium avium* complex confirmed by nucleic acid probes. They found that the recovery rates differed between the three methods, with ESP II recovering 71.2 % of mycobacteria compared to 63.9 % and 61.8 % on BACTEC MGIT 960 and Middlebrook 7H11 respectively. The increase in culture positive samples achieved in this study compared to others could also be due to the use of an updated protocol for the ESP *para*-JEM system. Rajeev *et al*. (2006) presented the results of a study to investigate this, in which they cultured 250 faecal samples for MAP. They investigated two methods of determining whether a sample was MAP positive. With the standard protocol, samples needed to have a gas positive signal from the system, a positive acid-fast stain, and a positive IS*900* PCR result. In the new protocol, all samples (gas-

positive and gas-negative) were acid-fast stained and IS*900* PCR, and could be classed as positive if either of the staining or PCR methods were positive. This group found that 15 % of the samples were classified as MAP-positive using the new culture protocol compared to only 6 % using the standard protocol. The second protocol in this investigation was most similar to that used in the cross-sectional survey discussed here which could be another reason why more animals were identified as MAP-positive using faecal culture than in other surveys.

Similar to the survey presented here, the survey in Ireland by Douarre *et al*. (2010) also observed differences in the number of faecal-positive animals detected between the farms they recruited, with only four herds having at least two MAP-positive cultures, even though all animals were recruited from studies who had a history of MAP infection. In this cross-sectional study, only three farms (A, B and E) had animals that were faecal culture positive, even though animals were found to be phage-PCR positive from the matched milk samples across all farms. While there were phage-PCR positive animals on all farms, milk culture positive animals were only found on the same farms as those with faecal culture positive animals, although only one animal was positive on both culture methods. Taking this into account, it could be suggested that there is asynchronous shedding between the milk and faeces, which has been seen by other authors. Gao *et al*. (2009) compared three milk detection methods (solid culture on HEYM, IS*900* direct PCR, nested PCR, and faecal culture) when used to test 99 matched milk and faecal samples taken across fourteen MAP-infected herds. All cows enrolled in that study had had a

previous positive result using one of the four tests and therefore the cross-sectional profile included more animals likely to give positive results. They found that there was no significant relationship between faecal culture and any of the three milk assays. This is similar to the results found in this study despite the fact that the animals in this study were not chosen according to their previous test history, but did come from farms with a known JD problem. Gao *et al*. (2009) commented that animals with high numbers of MAP in the faeces may not give a positive milk culture result at all, and vice versa. The result of this group agrees with the result seen in this study regarding asynchronous shedding in the milk and faeces.

The lack of association between milk and faecal culture methods may also be caused by intermittent shedding (Laurin *et al*., 2015; Mitchell *et al*., 2015) and other effects such as seasonality, lactation stage (Stabel *et al*., 2014), and age (Nielsen & Ersbøll, 2006). Mitchell *et al*. (2015) presented evidence from a study comparing the faecal shedding patterns of naturally and experimentally infected cattle in which they found that becoming a high shedder, and therefore having a higher probability of being detectable by faecal culture, is a rare and terminal event, with only 7 % of cows ever becoming high shedders. In contrast, naturally infected low shedders were seen to switch between no detectable shedding and low shedding, thus representing true intermittent shedders. They suggest that this provides evidence for the presence of multiple categories of immune response, and those animals who are intermittent shedders actually have infection under control. The findings presented by Mitchell *et al*. (2015) suggest that most of the cows within the

cross-sectional study presented here could be low shedders, and the reason for the difference in results between the milk-test and faecal-test results could be linked to the animals being in a period of no shedding.

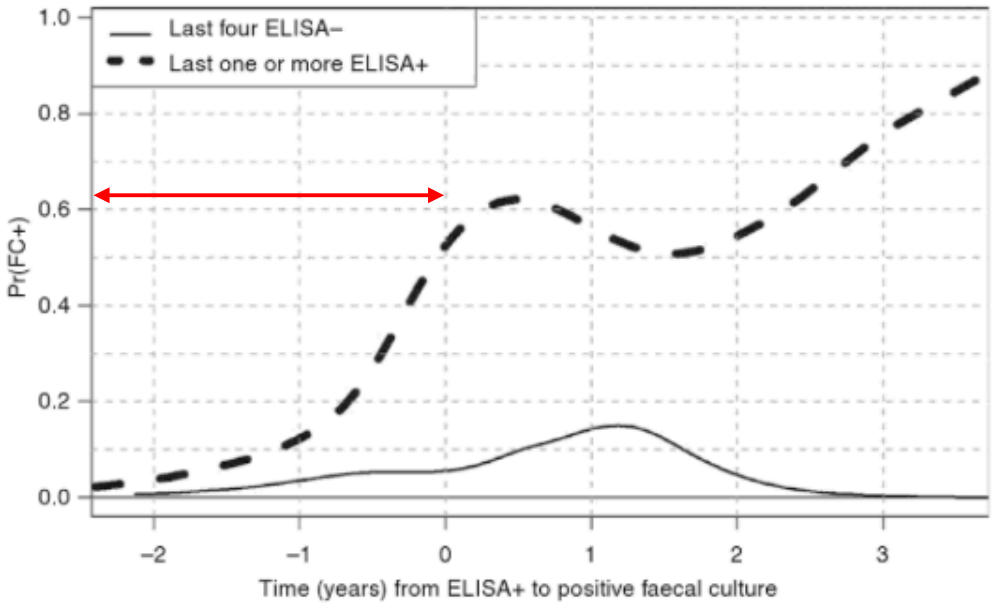
Laurin *et al*. (2015) also present data on intermittent shedding, by following a group of 51 naturally MAP-infected cows from seven Canadian dairy herds over one year, taking faecal samples monthly. Although no significant difference was found in faecal culture results between season and lactation stage, there were differences with respect to these factors in the results of direct qPCR of the faecal samples. In this case it was found that the probability of the qPCR test being MAP-positive in the summer and autumn were 85 % of the probability of samples from the same animals being positive in winter and spring. As samples from the cohort described in this chapter were taken January through to March, the same effect could be seen here. Laurin *et al*. (2015) also found that MAP shedding was higher during the dry-period, however no dry cows were included in the cohort studied in this chapter as it was essential to be able to collect milk samples for milk culture and phage-PCR.

In comparison to the culture methods used, the phage-PCR assay showed that 24.2 % of animals were shedding MAP into the milk, which is higher than the results of the initial survey detailed in Chapter 3 (19 %). The difference in prevalence could be due to the reduced number of cattle used in the second survey. It also identified more MAP-shedding animals than milk and faecal culture, suggesting that it has the potential to be more sensitive than the

other assays. The other consideration that must be given is that the phage-PCR assay does not have 100 % Sp and some of these animals could be false-positives. It is unlikely that this would have been caused by the test due to the phage element of the assay only identifying viable cells and the PCR detecting MAP-specific DNA. However, false-positives could have been caused by faecal contamination from the udder when collecting milk samples, even though care was given to reduce this risk.

The phage-PCR assay identified 28 % of faecal culture positive animals, of which all had an ab-ELISA negative history. Figure 4.3 adapted from Nielsen (2010), shows the probability of an animal having a positive MAP faecal culture result relative to a positive milk ab-ELISA test result (using a > 30 OD_{600nm} positive cut off). The dashed line shows that at the point when a cow gives a positive milk ab-ELISA test result, there would only be a 50 – 60 % chance that they would also give a positive faecal culture result within the first year after testing, the majority of cows would shed detectable amounts of MAP within 3.5 years. As highlighted on the graph, the area before this time-point between -1 – 0 and possibly even -2 – -1, is where the animals detected with the phage-PCR assay in the survey presented in this chapter would sit. This finding represents early detection of cows shedding MAP into the faeces, allowing removal of these animals sooner than if ab-ELISA was used alone.

Figure 4.3. Probability of testing positive with faecal culture relative to the time of testing positive with milk ab-ELISA



The probability of testing faecal culture positive is at approximately 60 % when an animal first tests ab-ELISA positive (positive cut-off of 30 OD_{600nm}). For the two years prior to the first ab-ELISA positive the probability of testing faecal culture positive is low. It is within this period of time that the phage-PCR assay may be providing early detection (highlighted in red; taken from Nielsen (2010))

In this study, there was a high proportion of animals who would not have been detected as MAP-positive if only one of the tests were used. A similar finding was also presented by Gao *et al*. (2009) who also found that there was a significant proportion of MAP positive animals that would have been missed if only one detection method was used, which led them to suggest that shedding in milk and faeces is not synchronised as discussed above. However, this also provides evidence for the argument that there is a need for multiple tests to be used to detect JD, as each test targets different aspects of JD and so combining all tests increases the ability to detect MAP infection at different stages of lactation and ages. Many authors have discussed the ability of different tests to detect MAP infection across a cows' yearly lactation and lifespan (Eisenberg *et al*., 2015; Nielsen *et al*., 2013; Nielsen *et al*., 2002; Weber *et al*., 2010). Nielsen & Ersbøll (2006) even suggested that younger milking cows should be tested more frequently than older cows as there was no apparent differences in the probability of testing positive with ab-ELISA by monthly sampling compared to testing every second year in older cattle. Although, it does have to be noted that this would substantially increase the cost of testing in a herd.

With regards to overall survey design, it could have been more informative to just look at animals who are already faecal culture positive. This would have allowed direct comparison to the ability of the phage-PCR assay to predict faecal shedding, and possible determination of Se and Sp. However, this would have significantly increased the cost of the study, since all animal in the herds would have had to be first tested by faecal culture which cost circa £42

per sample (SAC consulting, 2016). The funds available for this study only allowed 1000 cultures to be carried out across the two surveys. In a perfect situation, animals would have been screened with faecal culture, and only those who were positive selected to progress forward into this study. However, it does have to be noted that this would not guarantee that a group of animals would have been identified that were consistently shedding MAP in their faeces, as many animals will only intermittently shed MAP as discussed here. The other change that could have been made to the survey was the protocol for collection of faecal samples. Here, animals were observed in their natural environment and samples collected after they had defecated. One author found that of all animals tested, MAP was primarily isolated from milk samples of cattle within early lactation (0 – 60 days in milk; Stabel *et al*., 2014)), which is around the lactation period that the animals in a veterinary visit and for artificial insemination would be in. Another way to do this would have been to randomly select animals, run them through a race into a crush, and taking samples by rectal palpation. However, samples had to be taken without stressing the animals or making a change to their daily routine, so again this was undesirable.

In conclusion, the phage-PCR assay does not totally agree with either milk ab-ELISA, milk culture or faecal culture. However, it does seem to provide early detection of animals shedding MAP into the faeces when compared to milk ab-ELISA. This is the ultimate goal of any new JD diagnostic, as early detection allows infected animals to be removed from the herd at an earlier stage. Even

if this is not the case, it at least identifies some animals which are negative to other tests and may have been missed otherwise.

CHAPTER 5

PRACTICALITY OF USING PHAGE-PCR FOR LARGE SCALE FARM SAMPLING AND RECOMMENDATIONS FOR FUTURE USE

5.1 INTRODUCTION

Johne's disease is an insidious disease, made even more complicated by lack of Se in current diagnostics. Before they can be made commercially available, new detection methods such as the phage-PCR assay have to undergo rigorous evaluation to reveal any problems that might affect how the assay is used practically. Thorough validation by large-scale field trials will provide confidence in the results gained (G Hewinson pers. comms., August 2017). This is particularly important as the effect of individual animal or farm factors, such as the presence of other diseases, cannot be predicted by laboratory experiments. Jacobson (1998) stated that validation of diagnostic assays does not end with a short series of experiments on limited reference samples, but is a process that requires constant vigilance, maintenance and reassessment of its performance, especially when a new population of animals is targeted. There is evidence of this in the results gained during the experiments described in previous chapters. The results of previous experiments using phage-PCR in limited sample sizes of milk from individual animals (Stanley *et al*., 2007), blood samples (Swift *et al*., 2013) and cheese (Botsaris *et al*., 2010) did not highlight the problems that were revealed when larger scale testing of samples from individual animals across multiple farms was carried out.

It is well known that there is much variability in milk quality, as it is heavily influenced by the individual animal's health and the working routine of the farm staff (Leitner *et al*., 2000a; Quist *et al*., 2008; Syrstad & Ron, 1978). An example of this is the effect of differences between the feeding routine used

by two workers, as a badly prepared diet could contain differing levels of overall energy and protein which could influence milk solids (Linn, 1988). Milk quality and quantity can also vary depending on the genetics, age, stage of lactation and health status of the cow (Ng-Kwai-Hang *et al*., 1984). Forsbäck et al. (2010) suggested that knowledge on the day-to-day variation of milk composites is critical for the evaluation of milk data for any purpose, and therefore is equally important to consider when validating a new detection method that utilises the milk as the sample for analysis.

The somatic cell count (SCC) of the milk is a routine method used for monitoring the udder health of an individual cow, with increases in SCC indicating that the cow is at risk of mastitis. Somatic cells is a broad term that encapsulates macrophages, lymphocytes, polymorponuclear and epithelial cells (Leitner *et al*., 2000a; Lindmark-Mansson *et al*., 2006), with changes in the proportion of each depending on the health of the udder (Leitner *et al*., 2000b). It has been shown that there is a marked rise in somatic cells per millitre of milk during milking, with this level steadily declining throughout the day until subsequent milking (White & Rattray, 1965). Diurnal variation in SSC, *i.e.* changes detected between samples taken during morning and afternoon milking for the same animal, alongside changes in milk SSC associated with lactation stage, are both known to exist (Dohoo & Meek, 1982). This has important consequences for studies such as this as when the time of collecting samples cannot be standardised, especially if MAP is located within somatic cells, rather than free in the milk. Counts have also been seen to vary

seasonally, with SCC being lowest in May, rising through the summer months and peaking in December (Kennedy *et al*., 1982).

It has been well established that low-level faecal contamination of milk can occur during routine milking practices. This can provide a route of entry for MAP into raw milk. To provide evidence that the MAP detected by the phage-PCR assay represent cells that are shed directly into the milk rather than being introduced by faecal contamination, a comparison of the total viable count (TVC) of raw milk bulk tank samples and levels of MAP detected using the phage-PCR assay was undertaken by Botsaris, (2010). There was no significant difference in the mean TVC value of the MAP positive and MAP negative data sets. Therefore, the presence of MAP does not appear to correlate with the hygienic status of the milk, suggesting that faecal contamination is not the main route of entry of the MAP into the bulk milk. These results are in agreement with Corti & Stephan, (2002) who showed that there was no correlation between the total bacteriological count, somatic cell count and the detection of IS900 sequences by PCR in a survey of bulk tank milk and therefore faecal contamination alone cannot account for the presence of MAP in raw milk but suggests that these cells are being directly shed into the milk.

In the previous chapters the phage-PCR assay was used for large scale on-farm studies of milk samples. However, this was the first time that a large-scale trial had been conducted using the phage-PCR assay, so some problems were not unexpected. The aim of this chapter is to review the issues that arose and to discuss the ability of phage-PCR to detect MAP in individual animals when

applied on a large scale and how variation in mycobacterial load in the milk may affect detection of MAP. This information can then be used to inform recommendations for future use of the assay, both in laboratory scale research and in a commercial setting.

5.2 METHODS

5.2.1 Sample collection for longitudinal study

Milk samples are routinely collected using the parlour sampling systems, usually bottles attached to the milk line or shuttles on the robots (described in Section 2.4). In line with Farm A's routine testing protocols, samples were collected using the collection bottles in the parlour (as described and pictured in Chapter 2), on the same day that samples were collected by a commercial testing company for milk ab-ELISA. This series of farm visits were conducted as part of the longitudinal sampling of animals on the farm.

5.2.2 Environmental samples

Swabs from four areas of two milking robots and milk sampling machines on Farm Z (Chapter 2) were collected and tested for viable mycobacteria using the phage-PCR assay. The swab samples were collected from the robot between one cow being milked out and leaving the machine, and another entering. The robot is automatically rinsed between each cow, and washed fully once an hour, therefore samples were not taken following the full wash. The milk sampling machine was sampled when it was out of use, after it had been washed fully with hot water and detergent. Areas swabbed on the milking robots included the plastic tube from the milk jar to the sampling machine and the unit that attaches to the udder. Two other samples were also taken from the sampling machine, the spoon where the milk sample is collected before being put into individual pots and the plastic tube that leads

to the metal spoon (Fig. 5.1). The processing steps for the phage assay were modified for these samples by vortexing the MRD containing the cotton bud to release any bacteria, then the 1 ml of MRD retained for testing in the assay.

Environmental samples were collected from the milking machinery using sterile swabs and 1 ml maximum recovery diluent (MRD). The swab was dipped in MRD and the required area of the machine swabbed. The swab was then broken off into the MRD and taken back to the lab. Before testing with the phage-PCR assay (Section 2.5) the 1 ml MRD and swab were vortexed to release any bacteria on the swab into the liquid. The 1 ml MRD was then used directly in the phage-PCR assay.

5.2.3 Sampling during milking

To investigate changes in mycobacteria shedding during milking a small number of cows (seven) were systemically selected as they presented for milking on Farm A (every 25 cows) and a 50 ml sample taken from across the quarters by hand-milking before the clusters were placed on the cow. Further samples taken every subsequent two litres by removing the cluster and an equal sample taken from each quarter by hand-milking.

Figure 5.1. Location of swabbing areas for the environmental sampling of the robotic milking machines

1) tube to spoon
2) tube from machine to shuttle
3) spoon
4) cluster

5.3 RESULTS

5.3.1 Evidence of environmental mycobacteria contamination of milk samples in milking machinery

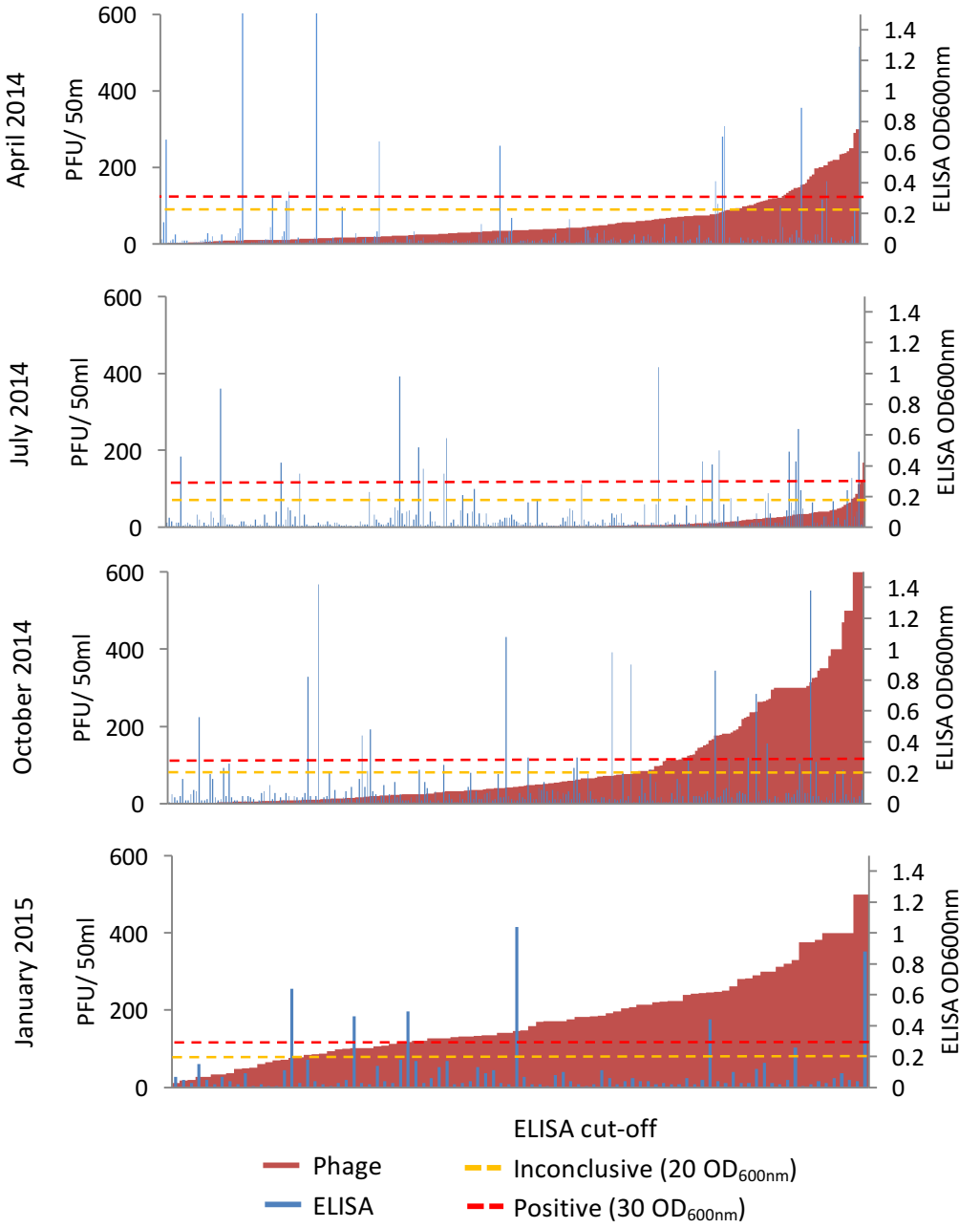
Over the first year of quarterly testing on Farm A there was found to be a marked difference between the shedding rates of the whole herd (Fig. 5.2; April 2014 to January 2015), with very few animals being phage-PCR positive (i.e. detection of other mycobacteria) at all time-points. With a range of 0-300 pfu 50 ml^{-1}, 0-201 pfu 50 ml^{-1}, 0-470 pfu 50 ml^{-1} and 0-500 pfu 50 ml^{-1} for the four time-points respectively. In isolation, these results were not surprising due to the number of cows that were ab-ELISA positive, or had an ab-ELISA positive test history, that were present on the farm. However, there was obviously some source of mycobacteria as very few animals were phage-PCR positive for MAP, even though mycobacteria were detected in the majority of samples tested. This was a concern, as it was unclear whether this was contamination of the samples with non-MAP mycobacteria within the milk, or failure of the MAP IS*900* PCR confirmation.

This was not an isolated incident, with samples taken from Farm Z also showing the same pattern of results. At this point two hypotheses were put forward to explain these results, the first suggesting that contamination of the milk with environmental mycobacteria from the parlour could be occurring, and the second that the cows were infected with another non-pathogenic species of mycobacteria. A third hypothesis, faecal contamination during

collection, was considered. However, this was ruled out due to the evidence presented by Botsaris (2010) on the lack of correlation between phage-PCR and TVC on bulk tank milk samples.

Failure of the IS*900* PCR to detect low level of MAP was also considered. Due to the presence of positive and negative controls using genomic DNA and *M. smegmatis* DNA extracted from plaques it was clear that the PCR was working, and no contamination was present. Genomic DNA was diluted 10-fold, stepwise to represent the copy number that was expected in a single MAP cell. Again, it was clear that although the bands on the electrophoresis gel were fainter when less DNA was present, the PCR was still able to detect these low levels.

Figure 5.2. Levels of mycobacteria detected for Farm A at the 4 different sampling points taken within a year.



The shedding rates of the cattle sampled (red bars, plotted from lowest to highest) are shown next to their matched milk ab-ELISA (blue bar). The herd-level shedding of mycobacteria differed between each of the four time points throughout 2014/15, with only a limited number of cattle being MAP-positive. As in previous chapters, ab-ELISA positive animals (above orange dashed line) had varying levels of mycobacteria in the milk.

Environmental samples from the milking robots on Farm Z were tested using the phage assay. Eight out of ten swab samples were found to contain viable mycobacteria (range 2 - 60 pfu per swab; Table 5.1), with one sample from the robots producing no plaques indicating that no mycobacteria were detected. Only one swab sample (spoon sample from robot 3) was found to contain MAP (Table 5.1), suggesting that other environmental mycobacteria exist within the robots and sampling machines. These were found in four sampling locations on two of the milking robots on Farm Z, suggesting that this is not an issue relating to one milking system. The detection of viable mycobacteria within the milking systems are surviving the between-cow automatic rinse conducted by the robot. However, the milk sampling machines were fully washed and out of use. The survival of mycobacteria suggests that mycobacteria could be surviving washing procedures by forming biofilms, which would affect the ability for phage-PCR to detect MAP when using these systems.

Table 5.1. Detection of Mycobacteria in the robotic milking machines by swabbing

Robot	Location	Phage assay (pfu ml^{-1})	Detection of MAP DNA in plaques by PCR
1	Plastic tube to metal spoon	7	-ve
1	Plastic tube from machine to shuttle	60	-ve
1	Spoon	3	-ve
1	Cluster	2	-ve
3	Plastic tube to metal spoon	0	N/A
3	Plastic tube from machine to shuttle	5	-ve
3	Spoon	15	+ve

N/A – PCR not conducted due to no plaques

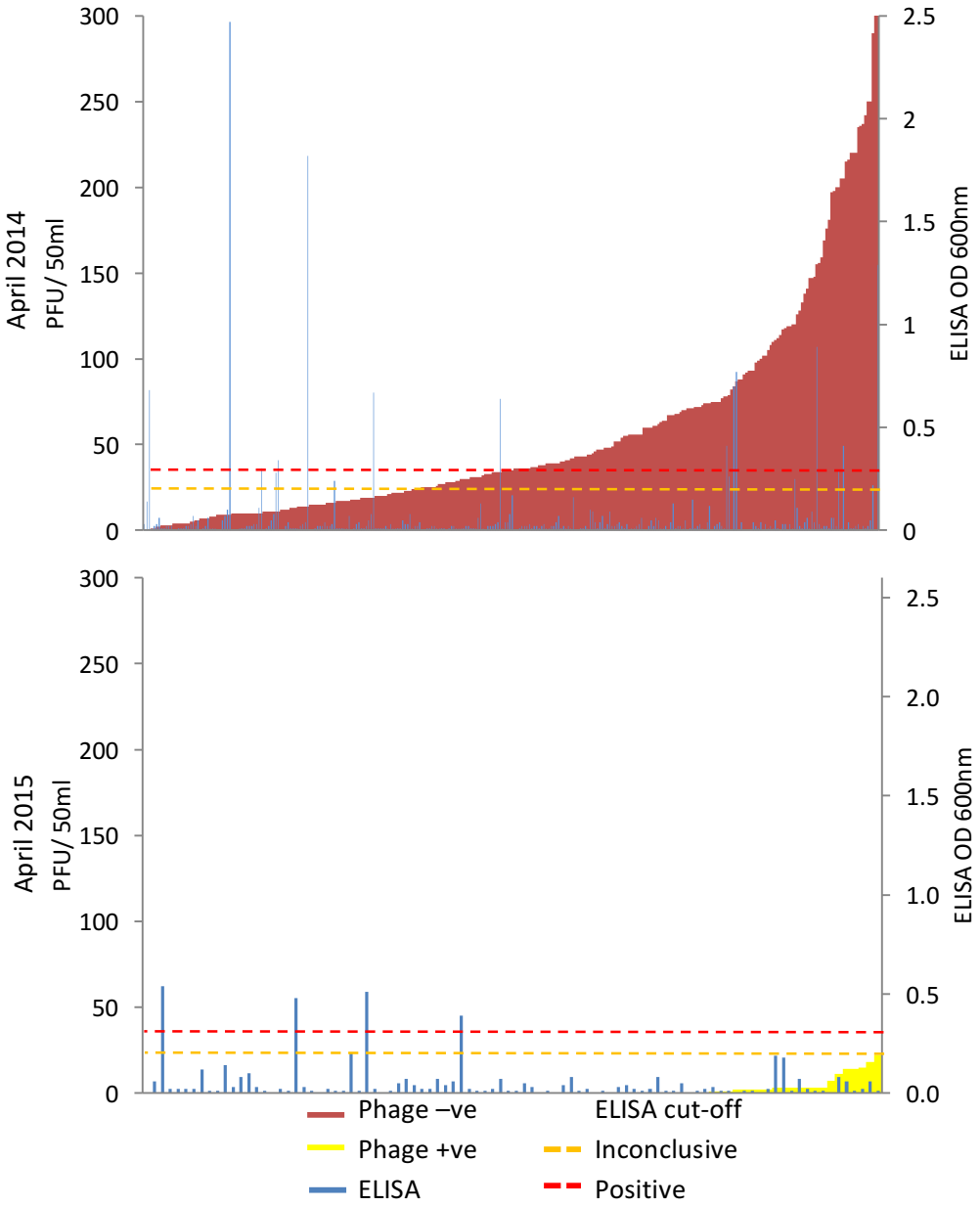
Mycobacteria were found across four sampling location in two robots on Farm Z. Only one sampling site was MAP-positive

To investigate whether the potential of biofilms in the equipment was affecting the results gained during the survey work, milk samples were collected by hand by systematically selecting cows when they presented themselves during milking from Farm A (every third cow; April 2015). The phage-PCR results of this time point (April 2015) were then compared to those from April 2014 to reduce the effect of seasonality on the detection of MAP as noted within the literature.

As can be seen in Figure 5.3, the apparent herd-level shedding rate of the cattle from Farm A decreased between the dataset from April 2014 and April 2015 (range 0-300 pfu 50 ml^{-1} and 0-28 pfu 50 ml^{-1} respectively). The number of MAP-PCR positive samples detected within the herd also increased (0 % compared to 23.4 %) when cows were hand milked in April 2015 compared to collection using the sample bottles attached to the milk line in April 2014. There were no management differences or changes to the phage-PCR assay between the two time points discussed here. Any cows that were removed from the herd were for reasons other than JD. The difference between the two datasets suggests that that environmental mycobacteria were present in the milking system due to the reduction in non-MAP mycobacteria and increase in MAP-positive samples when samples were taken via hand-milking.

Since Farm Z only used robotic milking machines, it was not used for any parts of the analysis since these cows were not accustomed to hand-milking and could not be hand-milked safely or without causing the animals distress.

Figure 5.3. Comparison between April 2014 and April 2015, with the samples for the latter taken by hand rather than using the milk sampling systems.



In April 2014 the apparent herd-level shedding rate was very high, with a limited number of cattle mycobacteria negative. When sample collection methods were changed in April 2015, the apparent herd-level shedding reduced, and 23.4 % of animals were MAP-positive with phage-PCR. There were no management or test changes between these time points.

5.3.2 Are non-MAP mycobacteria also present in the udder?

For each phage-positive sample in the cohort of animals detailed in Chapter 3 (553), fifteen plaques, or all plaques if there are less than fifteen, were picked and pooled into groups of five for extraction of DNA for PCR detection of MAP. All three replicates were tested for presence of MAP IS*900*, with a cow being declared as MAP-positive if one replicate was shown to have a band at approximately 200 bp when visualised (Chapter 2).

Interestingly, in a number of samples MAP DNA was only detected by PCR in one of the replicates (47.4 %), suggesting that low levels of MAP were present in each sample, with other plaques made up of environmental or non-pathogenic opportunists. Clearly in this case the plaque sampling protocol will affect the frequency with which a plaque-positive sample is determined to contain MAP, with those cows with a large shedding rate being less likely to be found to contain MAP cells.

In the cross-sectional study described in Chapter 3, 52 % of the animals that were plaque positive gave a negative MAP PCR result. It was also noted that there was a large range in the number of plaques detected, indicated that the number of other mycobacteria present in the samples was highly variable (1 to 544 pfu 50 ml^{-1}). However, there was no significant difference (p 0.440, variate: pfu 50 ml^{-1}, fixed: PCR result, random: farm) between the pfu 50 ml^{-1} results of the phage-PCR positive, compared to phage-PCR negative animals.

5.3.3 Variation in milk samples between the morning and afternoon and its effect on detection of MAP

Sample and collection variation is an important concept to take into account when undertaking analysis of any data. An example of this is the method for collection of milk ab-ELISA samples which has been described within the literature (Nekouei *et al*., 2015). This is particularly relevant when using the milk sampling bottles to take samples in the parlour since these are not cleaned or replaced between animals and sequential milk samples taken in the parlour have a risk of 'carryover' caused by the inclusion of residual milk from the previous cow in subsequent samples (Løvendahl & Bjerring 2006; Ordolff 1997). To accommodate this a cut-off value is set for the milk ab-ELISA results with samples giving a test result below the cut-off being classed as JD-negative.

As variations in the composition of milk during the day is described by multiple authors, it is important to investigate if this could affect the ability of phage-PCR to detect MAP. Therefore, it is crucial to know if shedding rates of mycobacteria can change during the day, so to identify the best time of day to take samples that will be most likely to give a positive result. To investigate this, cows were sampled during morning milking and afternoon milking (result reported in Chapters 3 and 4, respectively). The results for any animal that was sampled at both time points were gathered and used in this analysis. In total, 29 animals from Farms B, C and D met this criterion, and the mycobacterial counts for each of these time points were compared. There

was a significant difference between the number of mycobacteria detected in samples taken in the morning compared to those taken during the afternoon (p 0.001), with a sample taken during afternoon milking having a higher pfu 50 ml^{-1} of mycobacteria (mean of 7 pfu 50 ml^{-1} and 142 pfu 50 ml^{-1} respectively; Table 6.2). There was also a difference between the numbers of samples confirmed to contain viable MAP, with 28 % of animals MAP-positive in the morning samples compared to 16 % MAP-positive samples in the afternoon. Only one animal on Farm C was MAP-positive for both morning and afternoon samples. Since the afternoon samples generally had the higher mycobacterial load, it would be predicted on the basis of the results of Botsaris *et al*. (2013) that these would also be more likely to be MAP positive. This opposite trend highlights the fact that other types of mycobacteria are being introduced into the milk, and possibly in higher numbers in the afternoon. If this finding were generalizable to the whole population it may be advantageous to take samples in the morning to get a clearer picture of which cows are shedding MAP, without having result obscured to the introduction of other types of mycobacteria into the milk samples – from whatever route.

Table 5.2. Matched morning and afternoon milk samples

Farm	Line No	ab-ELISA (OD_{600nm})[a]	Morning Sampling		Afternoon Sampling	
			Phage	PCR[b]	Phage	PCR[b]
	752	7.06	19	0	0	
	1109	25.76	0		6	0
	1270	9.9	0		2	0
	1327	7.38	12	1	1	0
B	1266	4.74	2	0	0	
	534	2.85	24	0	319	0
	689	2.28	3	0	62	1
	497	7.64	3	1	185	0
	249	15.11	19	0	122	0
	715	3.19	3	1	152	0
	865	4.47	33	0	904	0
	492	3.08	1	1	66	0
	453	10.03	4	0	275	0
	883	66.7	27	0	642	0
	420	2.17	20	1	350	1
	235	8.29	18	1	377	0
C	991	10.89	2	0	107	1
	1298	6.23	3	1	47	0
	1322	6.11	3	0	43	0
	88	1.3	3	0	122	0
	138	521	0		53	0
	3280	3.3	2	1	53	0
	76	10.06	1	0	6	0
	9	6.39	0		3	0
	1227	16.23	0		10	0
	743	6.73	1	0	89	0
	912	12.16	0		0	
	1332	2.41	0		15	1
	859	1.42	3	0	94	0
D	28	2.79	0		11	0

(a) Red; positive, Orange; Inconclusive

(b) Red; MAP-positive, Green; non-MAP mycobacteria

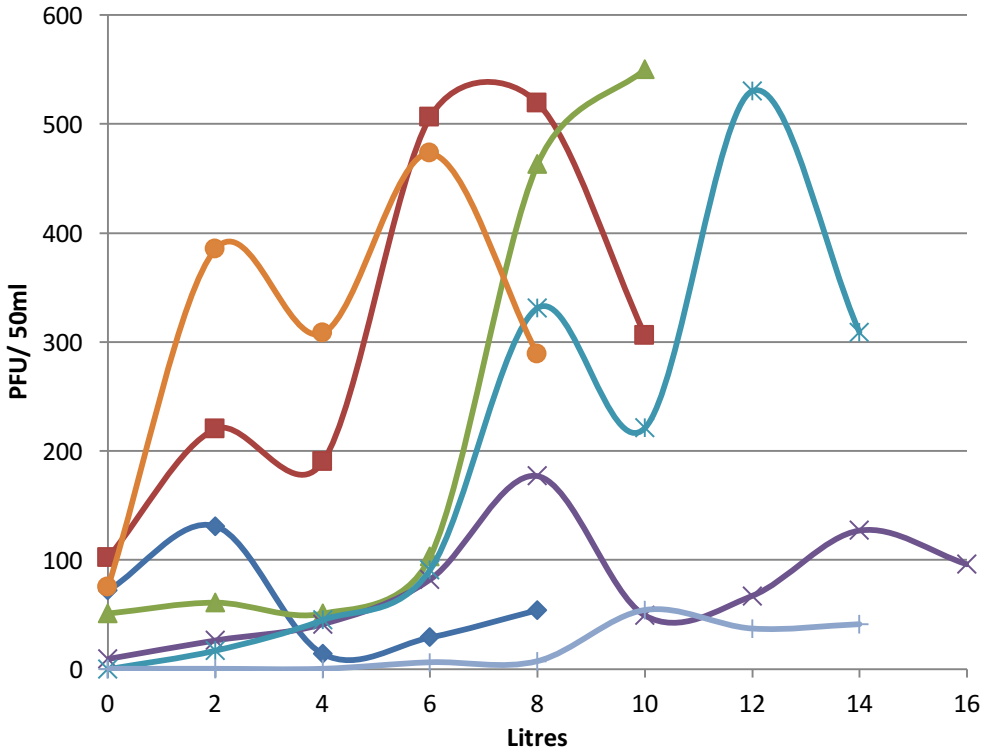
Morning samples have much less mycobacteria in comparison to samples taken in the afternoon, making it easier to confirm the presence of MAP with PCR.

5.3.4 Variation in levels of mycobacteria detected during milking

Another possible consideration is whether there is any variation in the number of mycobacteria that enter the milk during the actual milking process.

As can be seen in Figure 5.4, for all cows there was a general trend of increasing mycobacterial count being detected from the start to the finish of an individual milking observed. The most interesting pattern seen was where little or no mycobacteria was detected at the beginning of milking but by the end of milking the levels increased dramatically with the result for one animal increasing from 0 at the beginning of milking to 309 pfu per ml after giving 14 litres of milk.

Figure 5.4. Shedding of mycobacteria during milking for seven cows on Farm A



Seven random cows were sampled by hand before the cluster was attached to the udder (0 litres), and again every two litres until the cow was fully milked. Mycobacterial counts were determined using the phage-PCR assay, with all cows being PCR negative

5.4 DISCUSSION

The phage-PCR assay is a new method that can be used to detect and enumerate MAP in complex sample matrices such as milk and blood. There is a large amount of uncertainty the shedding rates of MAP in milk due to large knowledge gaps and therefore, there is little information about how this might change within and between milking. Forsbäck *et al*. (2010) suggested that day-to-day variations in milk quality could be caused by random biological variation, errors in the analytical methods, sampling technique and pathological and physiological changes, so it is prudent to investigate how these variations will affect a new detection method.

The main issue that became apparent during these trials was the variation in levels of non-MAP mycobacteria present in the milk samples. This causes two problems when reading results; first a cow can either be misclassified as high-shedding when she may only have a low level of MAP in the milk, and second this can mask the presence of MAP as there is a lower probability that a plaque originating from a MAP cell will be picked for PCR detection which affects the Se of the assay. Due to these factors, it is assumed that some of the plaque-positive, MAP-PCR negative samples are false-negatives, so the prevalence of 19 % across the six herds using phage-PCR may be an underestimation. However, it is interesting that there are other non-MAP mycobacteria in the milk, where it is generally accepted that the only other bacteria present will be fast growers such as *E.coli*, *Streptococcus* and *Staphylococcus*, which are well known causes of mastitis (Bradley, 2002).

Research into mycobacterial infections of the udder is limited, and generally restricted to older published cases from the 1970's where exact species confirmation was difficult. Mycobacteria is a very diverse genus, including many pathogens, non-pathogens and environmental species (Woods & Washington, 2011). The two most common species to be isolated from cattle samples are bTB and MAP, however many authors have isolated others such as *M. scrofulaceum*, *M. intracellulare* and *M. fortuitum*, from various matrices over the years (Cousins *et al*., 1999; Dunn & Hodgson, 1982; Franco *et al*., 2013; Shimizu *et al*., 1977), but isolation of these species have been mostly overshadowed by bTB and MAP due to their zoonotic potential.

The results presented in this chapter indicate that there is a risk of environmental contamination of samples when using milking robots. Non-MAP mycobacteria being detected in most areas of the milking robots sampled, including parts of the machines that would come into direct contact with the sample. There are multiple ways in which mycobacteria could be entering the system, with mycobacteria being present in the water used to clean the machinery (Dailloux *et al*., 1999; Le Dantec *et al*., 2002; Vaerewijck *et al*., 2005) and formation of biofilms with bacteria from the water or milk (Cook *et al*., 2010; Hall-Stoodley & Lappin-Scott, 1998) within the systems being the most likely sources of contamination. Many studies have found multiple species of mycobacteria in drinking water, with Covert *et al*. (1999) collecting 139 samples from five sources including public drinking water, cisterns, bottled water, water treatment plants and ice samples, across 21 states in the USA. Isolation was achieved by decontamination, filtration, then

culture on 7H10 Middlebrook agar slopes for eight weeks. Mycobacteria were isolated from 33 % of the samples, mostly from drinking water and treatment plants, with nine different species identified. A further study in the USA found that 135 of 528 samples from water distribution systems contained mycobacteria, where multiple slow- and fast-growing species were isolated (Falkinham *et al*., 2001). Le Dantec *et al*. (2002) conducted a similar study in Paris, utilising the same sampling techniques, and found that 72 % of samples from the water distribution systems contained mycobacteria, with the range of species identified including *M. gordonae*, *M. fortuitum*, *M. peregrinum*, *M. chelonae* and *M. intracellulare*. Dailloux *et al*. (1999) reported that *M. gordonae* and *M. flavescens* were the most common species found in water, and these were found to have some resistance to decontaminating agents (Dailloux *et al*., 1999), possibly explaining why they can be found in a system that is washed regularly like a parlour or robots.

All of these studies provide a possible explanation for the presence of mycobacteria in the milking systems, especially during washing. However, to be causing an increase in the subsequent milk samples they would have to be staying within the systems, most probably by forming a biofilm, which mycobacteria are able to do (Vaerewijck *et al*., 2005). Biofilms are defined as a group of cells that are irreversibly attached to a surface or each other, and embedded in a matrix of extracellular polymeric substances produced by the bacteria, making them more protected against disinfectants and other antimicrobial substances (Vaerewijck *et al*., 2005). Hall-Stoodley & Lappin-Scott (1998) theorised that biofilm formation in rapidly growing mycobacteria

could contribute to their resistance to disinfectant and mode of transmission, and found that *M. fortuitum* formed dense biofilms within 48 h of introduction to a laboratory-based, small scale circulating system, which pumped bacteria from a batch culture over 'studs' which could be easily removed for analysis at different time points. At the same time, a molecular method for the detection of mycobacteria within biofilms was also developed by Schwartz *et al*. (1998) who used this to show that mycobacteria were present in various drinking water systems using a 16S rRNA PCR on the biofilms. The mycobacteria identified were not found to belong to pathogenic species, but rather represented environmental species. However, there is evidence that MAP is also able to form biofilms, as demonstrated by Cook *et al*. (2010) through the testing of mixed-community biofilms from various livestock water troughs over one year. A glass tank was set up containing 32 litres of water collected from the drinking troughs from a farm and samples of the different trough materials (plastic, concrete, stainless and galvanised steel) were placed inside the tank to support the formation of biofilms. MAP was added at day zero, and the trough materials tested on day three and weekly for six weeks, then monthly thereafter. Quantitative qPCR was used to determine the concentration of bacteria, and MAP. They found that MAP was present on all trough materials throughout the study, with a concentration ranging between 7 % to 50 % of the total biofilm population. The findings by Cook *et al*. (2010) could present an issue when using the sample collection systems if MAP is able to form biofilms with the parlour, as cows could be misdiagnosed as MAP positive if the milk become contaminated with MAP in

this way. It is not thought that MAP biofilm formation is a problem for this study, as many more cows would have been classified as positive. The results of this study seem to indicate that it is other non-MAP mycobacteria that are causing the problem.

It has also been suggested that the parlour wash at the end of each milking could play a key role in the cleanliness of the lines (Blowey & Edmondson, 2010), and possibly the contamination seen in this study. Under normal circumstances a wash is conducted after every milking, and consists of three stages; pre-rinse, wash and post-rinse. However, multiple variables can affect the efficiency of the cleaning process which leaves the next samples of milk open to contamination. The first is insufficient use of chemicals which results in the milk residue and bacteria not be cleared efficiently from the lines. Since the microflora that is naturally present in milk includes both mesophiles and psychrophiles (Quigley *et al*., 2013) then these would be able to grow at ambient temperatures in the milking parlour leading to an increased risk of contamination for the following milk that passes through the machinery. Other problems include insufficient heating of the wash water, leading to incomplete inactivation of bacterial populations. However, if it is too hot, milk fat can be baked into the lines, creating a perfect environment for bacterial growth. In addition, defects in the machinery such as unstable or inadequate vacuum and dead-ends in the milk line will also reduce the efficacy of the cleaning procedures (Blowey & Edmondson, 2010). Since these are all recognised problems in milk production, the hygienic status of milk is monitored by milk buyers using the Bactoscan assay, an automated process

that provides an indication of the total number of bacteria present in the raw milk, with any increases being used to indicate potential problems in the parlour.

These risk factors that lead to the presence of significant levels of environmental mycobacteria within the milking machinery means that hand milking would be the preferred way to collect samples, rather than using the collection systems. However, this would put more pressure on the farms to carry out sampling due to the increased labour time required to hand strip every cow, and hence limiting the practical application of the assay. Another limitation for this sampling technique would be the increase in shedding of mycobacteria during milking. Sampling bottles attached to the parlour overcome this as they collect milk under vacuum, so a small amount is taken constantly, giving an even sample across the milking period. Sampling by hand at the start of milking will reduce the sensitivity of the assay, as this will cause an increase in false-negative results.

In addition to the random introduction of different environmental mycobacteria into the milk, a more directed process may be occurring. In the last decade *Mycobacterium vaccae* has been isolated from many environmental sources, but more importantly has also been isolated from udder and skin lesions of cattle (Franco *et al*., 2013), and there have also been many reports of mycobacteria causing Bovine Nodular Thelitis (BNT), a granulomatous dermatitis of the teats and udder (Thorel *et al*., 1990). Although uncommon, the isolation of *M. vaccae* (Shimizu *et al*., 1977), *M.*

terrae (Thorel *et al*., 1990) and species related to *M. leprae* and *M. lepromatosis* (Pin *et al*., 2014) from associated lesions means that this could also represent a source of mycobacteria other than MAP in the milk. Shimizu *et al*. (1977) described several cases of BNT in Japan where isolates resembling *M. gordonae* and *M. vaccae* were recovered from lesions of the teat, and used culture on Löwenstein-Jensen, Ogawa egg, Smith and 7H10 media to isolate colonies with the characteristic mycobacterial colony morphology which were also found to have the appropriate biochemical profile to confirm the identification. Even though this is an older piece of research, using more traditional identification techniques, more recent case studies are also available such as Pin *et al*. (2014) who discussed cases of BNT from a small herd of 30 cows, where at least one third presented with painless nodules on their teats. Histopathological analysis of biopsy samples revealed acid-fast bacteria in clumps free in the tissue and also within macrophages, which resulted in the diagnosis of BNT. The identity of the bacteria present in the biopsy samples as mycobacterial was confirmed by using 16S rRNA PCR, and on the basis of the sequence analysis, the previously unknown mycobacterial species was reported to be phylogenically similar to *M. leprae* and *M. lepromatosis* but belonged to a distinct branch, sharing ancestors with *M. hemophilum*. Limited other cases of isolation are also reported in the literature, but links to mycobacteria are still not proven due to the difficulty of isolation. The fact that it is still possible to identify unknown species of mycobacteria causing relatively well documented diseases in cattle illustrates our lack of understanding of this genus of bacteria. Even though BNT is worth

mentioning due to its links with mycobacteria and udder lesions, it is unlikely to have caused the number of phage-PCR negative samples recorded in this study. It is an uncommon infection and has only been reported in the literature in Japan, France and Switzerland (Pin *et al*., 2014).

There are limited references to mycobacterial-based mastitis, especially in cases with a history of failed treatment. Bercovier *et al*. (2001) published a review of the natural distribution of several mycobacteria within domesticated animals. *M. fortuitum* is thought to be a cause of chronic mastitis, and has been associated with large doses of intramammary antibiotics. A number of mastitis cases, described by (Whitney & Priddle, 1992) and (Wetzstein & Greenfield, 1992) highlight that *M. fortuitum* is an environmental opportunist, emerging after long-term antibiotic therapy. Two further authors have isolated this bacteria in raw milk, once in Tanzania (Kazwala *et al*., 1998) and again in Australia (Dunn & Hodgson, 1982). Outbreaks of mastitis have also been associated with *M. chelonae* (Menard *et al*., 1983) and *M. smegmatis* (Siqueira *et al*., 2016; Thomson *et al*., 1988)

It was interesting to see that higher mycobacterial counts were detected using the phage-PCR assay in the afternoon and at the end of milking compared to levels detected at the beginning of milking, and variation in SCC could account for this. White & Rattray (1965) reported that there was a 10-fold discrepancy in SCC between two samples taken for a feed intake study, the first being taken at 5 AM (just before milking) and the second being taken at 9 AM (three to four hours following milking). To investigate this further they selected four

cows with varying ages, and took 100 ml samples hourly (25 ml from each quarter), then manually counted the somatic cells. They found that there was a marked rise in somatic cells per ml of milk during and after milking, which fell before the next milking, hinting at diurnal variation. Even though the overall pattern was the same for all of the cows tested, the rise in SCC varied from 4 to 70-fold in individual quarters. These findings have since been corroborated by Olde Riekerink *et al*. (2007) and Syrstad & Ron (1978); the latter group reporting that SCC from morning milkings had 20 % lower SCC than samples from afternoon milkings. However White & Rattray (1965) also point out that the SCC recorded could also be related to a cow's yield, with those at the end of a lactation having the highest cell counts but the lowest yield therefore creating less of a dilution and thereby increasing the SCC recorded. The same could be said for daily milking routines, with the cell count being at its lowest just before morning milking, when the cow is giving its highest yield due to the longer overnight period between milking. However, whatever the reason for this variation in SCC, it is interesting that this pattern mirrors what was observed using the phage-PCR assay, with the pfu per 50 ml recorded being significantly lower in samples taken before morning milking than the levels detected in those samples in the afternoon ($p < 0.001$), and adds weight to the theory that the MAP cells being detected are inside somatic cells.

It is well accepted that MAP is found in macrophages, which form the predominant cell type making up 35 – 79 % of the total somatic cells in the healthy udder (Leitner *et al*., 2000a; Lindmark-Mansson *et al*., 2006).

However, the relative proportions change in an infected udder depending on pathogen, the stage of infection and the individual cow, with the proportion of macrophages decreasing for infections with *E. coli*, and PMN's and neutrophils increasing (Leitner *et al*., 2000b; Lindmark-Mansson *et al*., 2006). The effect of SCC on shedding does mean that before an appropriate testing regimen can be established when using the phage-PCR assays, the relationship with SSC needs to be fully understood so that a standardised testing protocol can be established to ensure that the results are truly representative of the infection status of the cow.

The phage-PCR assay has the potential to change the face of Johne's disease detection. It is rapid and improves on widely used diagnostics such as ab-ELISA, as it looks for the causative agent rather than an immune response which may be misleading. However, as highlighted in this chapter and those previous, there are a few issues that would need to be overcome to allow this to be used with greater effect. For research purposes, taking milk samples by hand to reduce the possible contamination of non-MAP mycobacteria from the milk lines is achievable. It is debateable as to whether this would be possible on farm as part of the milking routine, especially in large herds and with the increase observed in shedding rates during milking. This is something that would have to be developed further and may be overcome by using the six hour, one tube format described in Chapter 7.

The use of milk as a sample matrix comes with many problems, as it is not a sterile sample site and the sample can become contaminated with faeces.

However, this was found to not be a problem in this study as long as a clean sample collection protocol was followed, as outlined in Chapter 2, and if contamination was suspected samples were retaken or marked if the cow was unable to be resampled.

CHAPTER 6

A SURVEY OF RETAIL PASTEURISED MILK AND MECHANISMS FOR SURVIVAL OF MAP

6.1 INTRODUCTION

Alongside causing a significant health impact on the national herd, MAP may also have an economic impact on the dairy industry as a whole as it is thought to have an association with Crohn's disease (CD; Bull *et al*., 2003; Feller *et al*., 2007; Rhodes *et al*., 2014), an inflammatory bowel condition with similar aetiology to JD, making it a bacterium of potential zoonotic importance. Many studies have shown that MAP can be isolated from cases of CD, however it can also be isolated from controls and current techniques fail to detect the bacterium in all biopsy samples, so it does not fulfil all the criteria to prove an association with a disease as set out in Koch's postulates (Bach, 2015). However Feller *et al*. (2007) conducted a review and meta-analysis of 28 case-control studies, and concluded that the association between MAP and CD seems to be specific, but its role in the aetiology of disease still needs to be defined.

MAP survival in pasteurisation was first reported by Chiodini & Hermon-Taylor, (1993) using laboratory-based experiments designed to simulate both low-temperature long-time (LTLT) and high-temperature short-time (HTST) pasteurisation. They looked into this as they observed that in many CD studies the affected patients had no known contact with cattle or traditional sources of MAP, finding that 7.3 % to 39.3 % of MAP survived LTLT and 3.4 to 31.5 % survived HTST when stored at 4 °C after treatment. Millar *et al*. (1996) discovered MAP DNA by PCR from a small scale unit in the UK. This has been followed by Grant *et al*. (1996) finding that MAP is only consistently

inactivated when it was present at levels $<10^4$ cfu ml^{-1}. Grant *et al*. (1998) furthered this, finding that MAP could only be reliably inactivated at levels of 10 cfu ml^{-1} or 10 cfu per 50 ml, and was present in 14% of all other samples containing levels higher than 10^2 cfu ml^{-1}. These early findings led to most dairy processing centres adjusting the holding time of HTST pasteurisation from 15 s, which was the legal minimum, to 25 s, increasing the likelihood that MAP would be completely inactivated (Steele, 2000). Grant *et al*. (2005) continued to work on this, showing that MAP could be isolated from 44 % of samples after treatment at 72.5 °C, which is very close to that currently used in commercial plants. Despite all of this, McDonald *et al*. (2005) conducted multiple trials, combining the temperatures of 72, 75 and 78 °C with the holding times of 15, 20, and 25 s, in a commercial grade pasteuriser built for research purposes. They found that 85 % of the samples had a reduction of $>6\log_{10}$ and $>4\log_{10}$ in the other 14 %, by culture, concluding that pasteurisation was very effective at killing MAP in spiked milk. This level was backed up by Rademaker *et al*. (2007) who also found that a seven-fold reduction in MAP can be achieved. Due to this, these findings should be considered with caution as methodologies between laboratories varied, causing marked variations between results when replicated within different research groups as highlighted by Lund *et al*. (2002).

Clumping of cells has been suggested by many as a mechanism for survival of MAP in pasteurised milk as this would provide protection to a number of cells within the centre of the clump. Lynch *et al*. (2007) highlighted this in a series of experiments investigating the heat Se of MAP under pilot dairy-plant

conditions. They estimated the $D_{65°C}$-value of MAP to be around 20 s, which differed from previous estimations from Pearce *et al*. (2001) of 5 s. However, there were differences in growth conditions and treatment of cells before inoculation of the milk samples. Pearce *et al*. (2001) cultured MAP on a roller apparatus for up to 44 days to limit the amount of clumping, and performed homogenization in a sonicating water bath to reduce this further before inoculating into milk. In comparison, Lynch *et al*. (2007) grew MAP statically for 120 days and did not include a de-clumping step, with this technique exhibiting a high degree of clumping. Rowe *et al*. (2000) also looked into this by comparing a normal cell culture obtained by washing colonies from a slope to one that had been declumped by vortexing with glass beads for 2 min. The $D_{63°C}$-value for the clumped sample was 119.8 s compared to the declumped at 76.0 s. This phenomenon could be a factor in the differences observed in the laboratory-based experiments between research groups and between raw and laboratory-inoculated milk.

The detection of MAP has been historically difficult due to short falls in standard culture and PCR methods. Milk culture requires decontamination to eliminate fast growing bacteria, which in turn can decrease the likelihood of successful MAP detection as it causes a 1 to 2 $\log_{10}$ drop in cell viability (Dundee *et al*., 2001). PCR is more sensitive than culture, it will detect any MAP DNA present in the sample, whether this is from viable bacteria or dead cells, so although it is used extensively, it is not suitable to monitor survival. However, the phage-PCR assay only detects viable mycobacteria, identified by their ability to support the replication of Mycobacteriophage D29. Unlike

detection methods based on PCR alone, which just provide information on presence of MAP DNA, the phage-PCR method provides live-dead differentiation. Since D29 can infect all mycobacteria, this assay is combined with PCR of the end product to provide increased Sp. This element to the phage-PCR assay is key to its success, as it only detects live bacteria that have not been inactivated by pasteurisation, unlike PCR alone which will detect the presence of DNA whether the cells are alive or dead.

This assay has been used to detect viable MAP in raw milk (Botsaris *et al*., 2010; Stanley *et al*., 2007), powdered infant formula (Botsaris *et al*., 2016), cheese (Botsaris *et al*., 2010) and blood (Swift *et al*., 2013). The series of experiments described in this chapter primarily aimed to use the phage-PCR assay to estimate the prevalence of viable MAP in retail pasteurised milk. These were then extended to provide evidence for a mechanism of survival, whether this is heat resistance via clumping as described earlier by Lynch *et al*. (2007) or another method.

6.2 METHODS

6.2.1 Pasteurised milk survey

Milk samples (368) were provided by volunteers from their local retail supplier or doorstop provider at four time points across one year (May 2014, November 2014, January 2015, June 2015). A bottle of semi-skimmed milk (amount not specified) was purchased by the volunteers from multiple vendors within three counties across the East Midlands (Nottinghamshire, Derbyshire, and Leicestershire) and a 50 ml sample delivered to the laboratory within two days of purchase. Volunteers were asked to take a sample using tubes provided from a previously unopened bottle after upending to mix thoroughly, and record the supermarket, processor code and best-before date on the form accompanying the tube. Analysis of the information provided with the milk samples is not presented due to lack of compliance in recording and the limited number of MAP positive samples.

6.2.3 Survival of MAP

To investigate whether mycobacteria are free within the milk matrix or protected by cells as it is an intracellular pathogen, one litre of raw milk was taken from the bulk tank on Farm A before collection by the milk processor and split into twenty 50 ml samples after mixing thoroughly. Ten of these samples were processed as described in Chapter 2, termed whole samples. For the other ten samples, after the samples was resuspended in 2 ml M+

which lyses any somatic cells, the sample was split into two portions and each treated separately (split samples). Samples were then tested for the presence of mycobacteria using the phage-PCR assay.

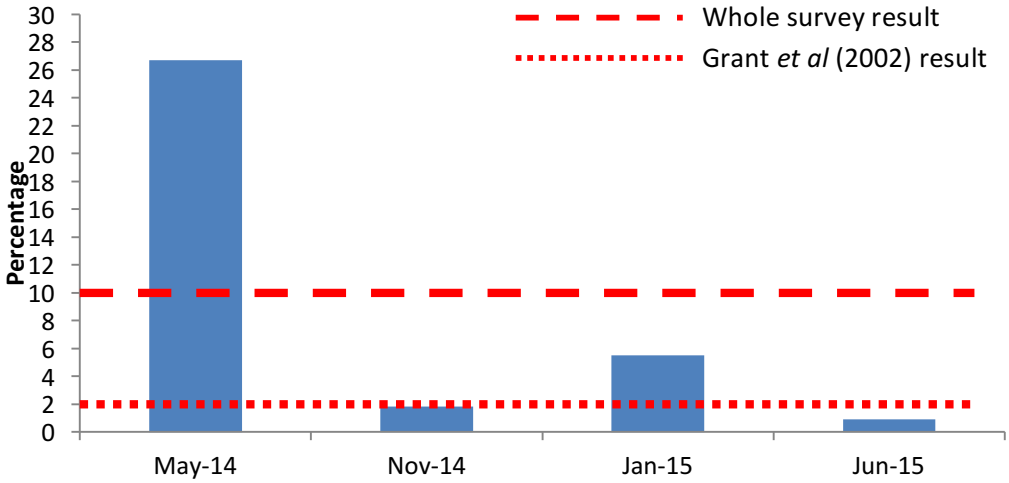
6.3 RESULTS

6.3.1 Detection of MAP in pasteurised milk

The phage-PCR detected viable mycobacteria in 32.34 % (119 of 368) of the pasteurised milk samples collected. When PCR confirmation was undertaken, 31.09 % (37 of 119) of these samples were confirmed to contain viable MAP cells. Overall, 10.33 % of the pasteurised milk samples collected were positive for viable MAP cells, an apparent increase from the last UK survey conducted in 2002 (Grant *et al*., 2002). Figure 6.1 shows the distribution of the MAP-positive samples and how this changed across the four sampling dates, in comparison to the average found within this study and that reported by Grant *et al*. (2002) using culture to detect MAP.

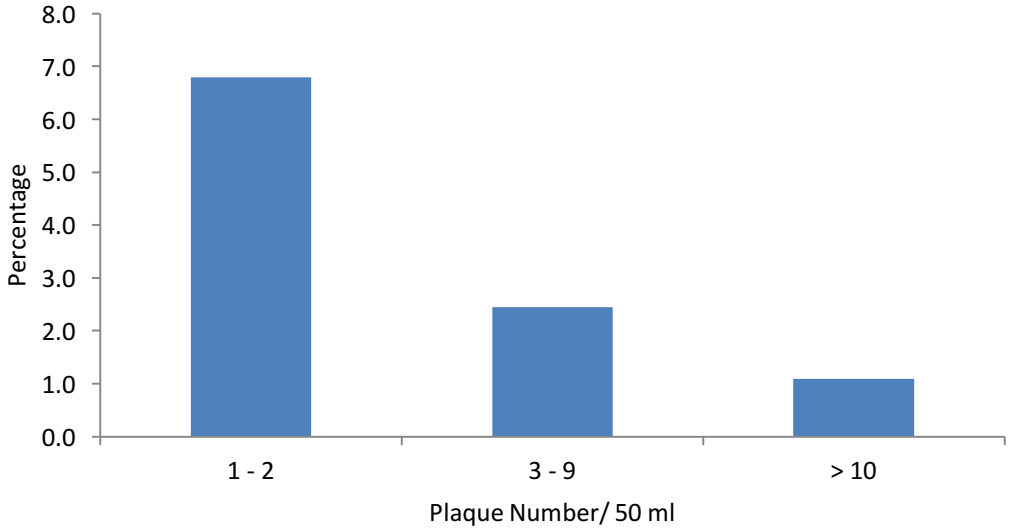
The survey results can be broken down further, by analysing the number of cells detected in each sample. Figure 6.2 highlights those groups that could theoretically be detected by PCR (> 1-2 cells per sample) and culture (>10 cells per sample). More than 10 cells were detected in only 1.1 % of the samples, and only these would have been theoretically detectable by culture after decontamination due to the drop in viability caused by chemical decontamination. A further 2.4 % of samples contained levels of cells theoretically detectable by PCR. In total, 6.8 % of the samples contained levels that would not be detectable by any other method currently available for MAP detection, showing a possible 10-fold increase in Se compared to other surveys that have been conducted in the UK. However, this increase does not represent an increase in incidence, only an increase in Se.

Figure 6.1. Percentage of positive samples for each sampling point



The overall percentage of positive samples (dashed line; 10.33 %) and the result from the Grant *et al* (2002) survey (dotted line; 1.8 %) also represented. Grant *et al* (2002) was chosen for this example as it was the last survey to be undertaken within the UK, other surveys are discussed within this chapter

Figure 6.2. Plaque number of MAP-positive samples



A total of 38 MAP-positive samples were detected, defined as those that produced plaques and were IS900-PCR positive. Data was grouped by plaque number per 50 ml sample and the results for each group reported as percentage of the total sample set (n = 368 semi skimmed (1.7 % fat) milk). The groupings were based on the reported sensitivities of other methods i.e. 1-2 cells; no other method reports being able to detect this low level, 3-9 cells and >10 cells; potentially detectable by direct PCR; >10 cells; potentially detectable by culture.

6.3.2 Survival of Mycobacteria from commercial pasteurisation techniques

Figure 6.3a shows the results for the whole and split samples described in 6.2.3. The whole samples have a large variation in the mycobacterial counts, ranging from 16 to 145 pfu viable cells, and when randomly paired are not well correlated. However, the paired split samples are positively correlated in comparison to the whole (r^2 = 0.78; Figure 6.3). The mean pfu was 50 and 49 for the whole samples and split pairs respectively, with the paired samples when treated separately having a median of 23 (approximately half of the whole samples), as shown in Figure 6.3b. The pfu range was very similar for the two groups, with the whole samples having 16 to 145 pfu, and the paired from 13 to 131 pfu (5 to 74 when A and B taken as separate samples).

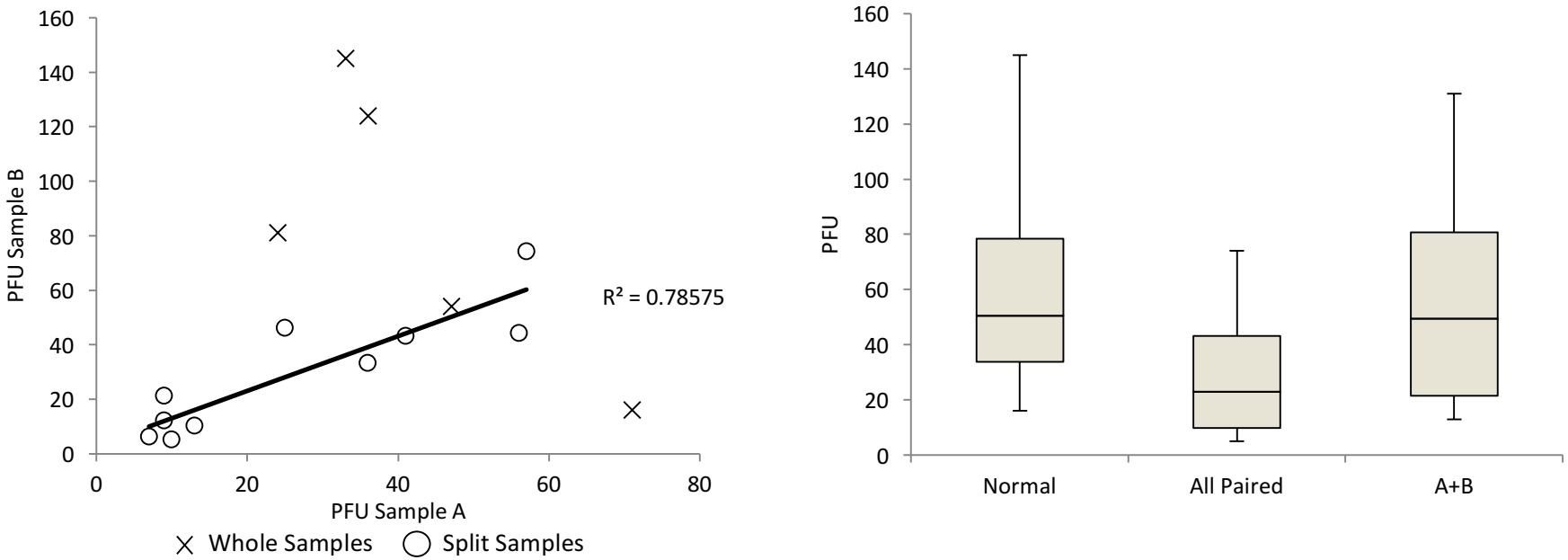
Two further analyses which examine a null hypothesis of complete spatial randomness (CSR) when samples are split into subsamples. The first examines the number of mycobacteria present in each of the 50 ml samples, including the counts formed by summing the counts in each of the two paired samples, giving twenty samples in total. The second uses a permutation test to compare the total mycobacterial count in the paired samples with that expected from a random (binomial) allocation. To analyse the initial allocation into 50 ml subsamples, a Pearson Chi-squared test is used, in which there are 19 d.f and the expectation of each sample is equal to 59.4, which is the total count in the whole sample divided by twenty. This test essentially assumes that the counts in each subsample are drawn from a Poisson distribution with

mean equal to 59.4. The result is that this null hypothesis of CSR across the subsamples is strongly rejected ($p<0.001$). These results can be explained if the distribution of mycobacteria present in the bulk tank milk is uneven so that when an individual 50 ml sample is taken there is likely to be a significant variation in the numbers of mycobacteria detected. The second allocation into paired samples was analysed using a permutation test. Expressing each of the ten allocations as a proportion $p_i=N_{1i}/N_{Ti}$ of the total $N_{Ti}=N_{1i}+N_{2i}$ of the two halves for that sample, generating 10,000 sets of equivalent proportions from a binomial distribution. Each of these sets takes the form $p_i^*=r_i/N_{Ti}$ where $r_i \sim B(N_{Ti},0.5)$ for i=1,…10, thus being a random allocation of the same total count as the corresponding sample. For each of these sets, a summary statistic, in this case the variance of these proportions, in calculated. The position (order statistic) of the variance of the proportions from the data within this list of 10,000 variances from the binomial allocations, gives the p-value. As a two-tailed test needs to be used, the smaller of the two order statistics is doubled to obtain our final p-value, $p=0.625$, so cannot reject the null hypothesis of randomness.

The only difference between the two treatments was the lysis of the somatic cells to release any intracellular mycobacteria before the paired samples were split (Fig. 6.3). Evidence of clustering is seen in the initial splitting into the twenty 50 ml samples, this is no longer present after this lysis step has been carried out. This pattern could be explained if the mycobacteria in the original milk were inside somatic cells rather than being free in the milk matrix,

explaining the heterogeneous distribution of MAP cells in the 50 ml milk samples.

Figure 6.3. Correlation of paired sample of bulk tank milk



Mycobacteria are thought to survive pasteurisation because they are protected from heat. One litre of raw milk was taken from a local farms bulk tank and split into 20 x 50 ml samples. These were then either processed as a whole (ten), or split in half after lysis of the somatic cells (ten).

6.4 DISCUSSION

It is well-known that viable MAP can be found in pasteurised milk in varying levels (Ayele *et al*., 2005; Ellingson *et al*., 2005; Millar *et al*., 1996; O'Reilly *et al*., 2004). Therefore, it was not surprising to find that 10.3 % of retail pasteurised milk samples contained viable MAP. This study was conducted across one year, with four sampling time points to remove the effects of seasonality suggested by Millar *et al*. (1996) who saw surges in prevalence in January to March, and September to November, and Grant *et al*. (2002) who saw apparent increases in late Winter and early Spring. As can be seen in Figure 6.1, the proportion of MAP-positive samples taken in May 2014 is much higher (27 %) than at others (1 % - 6 %), with this range in prevalence echoing the direct PCR results from Grant *et al*. (2002; 10 % average, range 0 – 27 %). However, to gain further information on the seasonality of MAP survival in pasteurisation a longitudinal study with more time points is needed. The results seen here show an apparent rise in the prevalence of MAP within pasteurised milk, compared to the last UK studies using a culture-based method (Grant *et al*., 2002; 1.8 %; O'Reilly *et al*., 2004; 0 %). However, it must be noted that this does not necessarily denote an increase in prevalence, but rather an increase in test Se as phage-PCR overcomes decontaminations issues and has a lower detection limit than other methods.

The phage-PCR assay has been proven here to be a useful tool in the detection of the low levels of MAP found in pasteurised milk, particularly as it is rapid and relatively low cost. The key element of the phage-PCR is that it

allows enumeration of MAP rather than just detection. This gives advantages over automated culture and PCR, which will only report on presence or absence and does not readily provide enumeration. Figure 6.2 shows the distribution of plaque number detected in all MAP-positive samples. Only 1.1 % of MAP-positive samples contained more than ten detectable cells (range 10 – 32). It has been shown by many that the chemical decontamination methods needed for successful culture work also reduces the number of MAP cells detectable by at least 1 – 2 $\log_{10}$, giving a low limit of detection (Bradner *et al*., 2013; Dundee *et al*., 2001; Grant & Rowe, 2004), making it a slow and insensitive way to detect MAP (Dundee *et al*., 2001). Culture has a Se of between 5 - 70 % depending on the decontamination method used, but Sp is usually assumed to be 100 % (Nielsen & Toft, 2008), so any positives are true-positives. This means that it is unlikely that samples containing less than ten MAP cells would give a culture positive result in the previous studies. Given that the other published surveys of pasteurised milk report viable MAP in 1 - 2 % of samples, the results gained in this work are in line with previous studies. Detection using direct PCR is thought to be more sensitive than culture, as this method has a lower detection limit. The Se of MAP PCR is 4 - 76 % (Bolske & Herthnek, 2010), depending on the microbial load and the sample matrix, but provides a Sp of over 99 %. In this survey 3.5 % of samples contained more than two cells (1.1 % > 10, 2.4 % >3 – 9) so would be theoretically detectable by direct PCR using the detectable limit estimated by Logar *et al*. (2012) of around 2 cells per sample. This is lower than other published surveys using direct PCR, which have reported MAP-positive prevalence's between 7 – 12 %

(Grant *et al*., 2002; O'Reilly *et al*., 2004). However, direct-PCR methods detect all DNA within a sample, whether this is from a live or dead cell and the phage-PCR method only detects DNA from viable cells, accounting for the lower levels detected. This means that it could be counterproductive to use direct PCR to estimate the survival of MAP in samples that have been processed using a method that aims to inactive the bacteria. Any prevalence estimated by PCR will therefore be an over estimate as it will include all bacteria that have been inactivated as well as those that are alive. The rest of the MAP-positive samples (6.8 %) are within the 1 – 2 viable MAP cell range, which is below the detection limit of other methods, so this group represents those who would not normally have been detected.

Grant *et al*. (1996b) has previously reported that after an initial drop in viable MAP there is a 'long tail' in the inactivation curve whereby a low number of viable MAP cells in a sample survive, even after heating for an extended period of time. Grant *et al*. (1996c) studied the thermal inactivation of multiple different types of mycobacteria (*M. avium, M. bovis, M. fortuitum, M. intracellulare* and *M. kansaii*) in milk at 63.5 °C. Survivors were enumerated after heating for 0, 5, 10, 15, 20 and 30 min and thermal death curves were constructed for each species. *M. bovis* and *M. fortuitum* were found to exhibit linear thermal death curves and neither species demonstrated any survival after heating at 63.5 °C for 30 min (equivalent to holder pasteurization). In contrast, *M. avium, M. intracellulare* and *M. tansaszi* yielded thermal death curves which exhibited significant 'tailing' and all three strains survived holder pasteurization. This type of kinetics indicates

that there is a heat-resistant sub-population, and would be consistent with our observation that the largest number of samples contained only 1-2 detectable MAP cells. One explanation for this phenomenon could be if the MAP cells were internalised within somatic cells which provided some protection against heat inactivation.

The majority of samples within the set tested here were pasteurised using the traditional HTST method. However, some samples were filtered products (n = 15), which remove more organisms by microfiltration using fine ceramic filters before conventional pasteurisation with HTST. This combination, along with bottling in an opaque container, provides an extended lifespan of up to three weeks (Arla, 2017). Surprisingly, one of these samples was positive for MAP (3 pfu $50ml^{-1}$), with a further eight containing mycobacteria (range of 1 – 32 pfu $50ml^{-1}$). MAP in filtered milk has been looked at before, for instance a study by Gao *et al*. (2002) contained a small portion of this type of milk (31/710). None of these samples were positive for MAP via PCR, however this could be due to low numbers of MAP below the limits of detection. Due to the small sample size in both studies, conclusions cannot be drawn from this, however a matched study of filtered to non-filtered milk would be interesting.

MAP has been linked to CD for many years, with Feller *et al*. (2007) confirming that the association seems to be specific with a meta-analysis of 28 case-control studies, but its role in the aetiology still needs to be defined. One of the issues is that Koch's postulates has still not been met for the disease (Bach, 2015), as MAP cannot be reliably isolated from cases, and has also

been isolated from controls and other irritable bowel syndrome (IBS) sufferers. A study by Scanu *et al*. (2007) highlights this issue well, finding that MAP could be isolated via IS*900* PCR of mucosal biopsy specimens from 75 % of IBS patients, 87 % of CD patients, and 15 % of heathy controls. Many others including Bull *et al*. (2003) and Naser *et al*. (2004) have similar findings. The apparent increase in prevalence in pasteurised milk could provide an explanation as to why MAP can be isolated from control patients and other non-CD patients. There is also evidence that the incidence and prevalence rates in Asian populations is increasing (Goh & Xiao, 2009), which could be linked to their increase in dairy product consumption (Dong, 2006; Whaley *et al*., 2003).

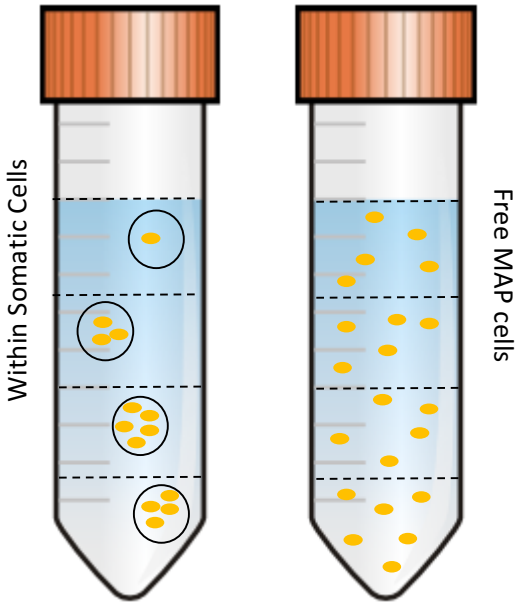
There was also a proportion of samples (32.8 %) found to contain general mycobacteria in the survey. This is not surprising considering the results from the survey of raw milk across six farms in the UK (Chapter 3), where 52 % of the cattle tested were PCR-negative following plaques being detected using the phage assay (contained non-MAP mycobacteria). Dunn & Hodgson (1982) isolated this mycobacteria, amongst others, in raw milk from pasteurisation plant storage tankers, farm pick-up tankers and farm bulk tanks using the same decontamination and culture methods as Kazwala *et al*. (1998). However, they were unable to gain any isolates from the pasteurised milk, suggesting that it is heat sensitive. This was corroborated by Grant *et al*. (1996) who studied the thermal inactivation of numerous *Mycobacterium* at 63.5 °C using inoculated raw milk containing approximately 10^7 cfu ml^{-1}, of

which *M. fortuitum* was found to be the most heat sensitive, with no recovery after ten minutes.

Routes for entry of non-MAP mycobacteria into raw milk were discussed further in the previous chapter.

The viable MAP count (via the phage-PCR assay) varies vastly within 50 ml samples of raw bulk tank milk (range 16 – 145). This suggests that MAP is not equally spread and free throughout the milk, as all samples would be expected to have a similar number of cells present if they were free to diffuse due to being mixed once in the bulk tank, and again before aliquoting. When taking the marked differences between the results of the split samples and whole samples into account this confirms that MAP cells are contained within another cell. The only difference between the two treatments was the lysis of the somatic cells to release any intracellular mycobacteria before the paired samples were split. There is clear evidence of clustering in the initial splitting into the twenty 50ml samples, which is no longer present after the lysis step has been carried out. This pattern could be explained if the mycobacteria in the original milk were inside somatic cells rather being than free in the milk matrix, explaining the heterogeneous distribution of MAP cells in the 50 ml milk samples (Figure 6.4).

Figure 6.4. Schematic diagram of somatic cell loading of MAP, in comparison to it being freely distributed in milk.



The results of the retail pasteurised milk survey and other published surveys discussed here, show that MAP is able to survive pasteurisation, with the second experiment suggesting a mechanism for survival. Laboratory studies that simulate pasteurisation of MAP-spiked milk have consistently shown that HTST pasteurisation conditions are effective at reducing the levels of viable MAP by 3-6 $\log_{10}$ (McDonald *et al.*, 2005). Levels of MAP reported in raw milk from infected cows are not thought to be more than 10^2 cfu ml^{-1} (Slana *et al*., 2008; Sweeney *et al*., 1992), so pasteurisation should achieve complete inactivation of MAP. However, these experiments use laboratory grown bacteria that are added to milk, so free in the matrix. The experiments contained within this chapter suggest that MAP in raw milk is located within the somatic cells (made up of polymorphonuclear leukocytes, macrophages, lymphocytes and a small number of mammary epithelial cells; Boutinaud & Jammes, 2002). It has previously been shown that MAP surviving inside amoeba are approximately two times more resistant to chorine disinfection (Whan *et al*., 2006) and this is due to the protection provided by the large mass of the host cell surrounding the bacteria (amoeba = 15-35 µm). Protection against chlorine disinfection has also been reported for a range of internalised bacteria, especially when they are located within vacuoles (King *et al*., 1988), with Bannantine & Stabel, (2002) explaining that MAP can be found inside vacuoles of infected macrophage. Somatic cells in milk generally range in size from 10-20 µm, and so a similar size to the amoebae used in the above disinfection studies. It is therefore possible that the survival of low levels of MAP during pasteurisation is due to their intracellular location, with

the somatic cell providing sufficient protection to prevent complete inactivation. Patel *et al*. (2006) showed that bovine mammary epithelial cells contain up to 10^4 MAP cells, and MAP are known to be able to replicate within macrophage (Arsenault *et al*., 2014), so it is likely that infected somatic cells will not contain single MAP cells, increasing the likelihood that the low numbers of survivors could represent a residual population of survivors that have been physically protected from the heat treatment.

Another suggested route into milk is low level faecal contamination during milking, with these MAP cells thought to be free within the milk matrix. Botsaris (2010) conducted an experiment that compared the total viable count (TVC) of raw milk samples and levels of MAP detected using phage-PCR. He found there was no significant difference in the mean TVC value of the MAP-positive and MAP-negative samples, so the presence of MAP does not appear to correlate with the cleanliness of the milk, suggesting that faecal contamination cannot be the main route of entry into milk. The experiments described in this chapter agree with the findings of Botsaris (2010), and agree with the idea that MAP is not free within the milk. Free cells within the milk, as seen with laboratory spiked samples, will be more susceptible to heat treatments, so will be inactivated easier than those cells found within somatic cells, so protected from the heat, allowing them to survive any heat treatment.

Since each plaque forming unit represents one viable mycobacteria in the phage-PCR, it allows enumeration alongside detection. This element of the

assay could prove useful for dairies as they will be able to not only compare the numbers of mycobacteria in the raw milk and their pasteurised product, but also the cleanliness of the plant. Mycobacteria have the ability to form biofilms (Wu *et al*., 2009) and have been demonstrated by many, with Cook *et al*. (2010), showing the survival of MAP in livestock watering trough materials, which has been discussed in Chapter 5. Since MAP readily form biofilms, and has been suggested from an experiment contained within Chapter 5, another route of entry into pasteurised milk is from possible biofilm formation within the pasteurisation plant. This means that post pasteurisation contamination is also possible. Environmental testing of plants represents another use for the phage-PCR assay beyond the testing of clinical and food samples.

The association between MAP and CD has been discussed for many years (Feller *et al*., 2007; Rhodes *et al*., 2014), with pasteurised milk thought to be a key vehicle of transmission to humans. This series of experiments highlights that the prevalence of MAP in retail purchased pasteurised milk is higher than has been previously quoted due to improvements in test Se enabling low levels of MAP to be detected. There is potential for the food industry to use this method to monitor the efficacy of pasteurisation processes, as it is rapid and relatively low cost, alongside monitoring cleanliness in the plant. Although a potential method for survival has been suggested here, there is a need for further research to fully understand this and how it will affects current pasteurisation processes.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The results described in this thesis have shown that the phage-PCR assay has the potential to improve JD diagnosis by both identifying more infected animals. It could do this by providing early detection in the shedding curve therefore reducing the overall MAP load within the herd. Johne's disease has a significant financial impact on the health of the national herd through losses caused by a reduction in milk yield, increased feed costs, loss of value in breeding stock, earlier culling decisions and a loss of money from a decrease in killing weight (Barkema *et al*., 2010; Lombard, 2011). Currently, due to the lack of Se and Sp of the main diagnostic used within the UK, only a small proportion of infected animals are captured. This is even less if only clinical cases are considered due to the so called iceberg effect of latent infection (Magombedze *et al*., 2013). If detection rates were to be improved, making it possible to find as little as one MAP cell per sample, in theory, every single animal that is shedding into milk could be detected.

The results presented within Chapters 3 and 4 suggest that phage-PCR could be more sensitive than milk ab-ELISA, milk- and faecal culture, by detecting more MAP shedding animals than the other methods in both of the cross-sectional surveys presented here. There is a lack of agreement between the four detection methods discussed throughout Chapters 3 and 4, so if phage-PCR is not more sensitive, it could at least detect extra animals beyond those identified by a single test. This provides evidence to advocate the use of multiple tests within a control programme, much like the use of the SICCT as primary detection and IFN-γ as secondary detection for the control of bTB in the UK.

Investigations into how the milk ab-ELISA history of a cow effects the MAP shedding level detected by phage-PCR proved that those who have had a positive ab-ELISA result within the last year (>20 OD_{600nm}), no matter how high over the cut-off this was, were shedding less MAP than those who had MAP-negative history. This suggests that a single immune response could be the animal controlling the disease in the short-term. There was no difference in the shedding rates between those animals with an ab-ELISA positive history and those with an ab-ELISA negative history if the ab-ELISA test positive was over one year ago. The second survey, comparing faecal culture to phage-PCR, found that phage-PCR identified 28 % of faecal culture positive animals who had no ab-ELISA positive history. This suggests that it could be providing earlier detection than ab-ELISA.

There is a consequence of improving the tests to this level of Se, specifically the question of what would be the fate of all of the additional animals identified as being infected. As it stands, even the improved Se of the phage-PCR method is potentially only detecting those animals that are very high-level shedders, that have been shedding into the herd for a number of years, so it is an easy management decision to remove them from the herd to improve disease control. However, if all infected animals could be detected, no matter how little they were shedding, a cost-benefit analysis would be required to determine how they would be dealt with. One downside of finding more infected animals within a herd would be increased costs, especially when using a test-and-cull strategy. Kudahl *et al*. (2007) described how herds would benefit from the improvement of tests over a 10-year period. However,

it would be more expensive to use than the current test in the first five years due to the drastic increase in replacements needed. However, this would only be an issue immediately following the change in Se. After this point, the number of test-positive animals would reduce as long as appropriate management practices were adopted to stop transmission to young stock. The net annual revenue per cow would then increase continuously.

Certain infectious diseases are notifiable, this means that any veterinary practitioner who makes a diagnosis of one of these diseases is obligated by law to inform the Animal and Plant Health Agency (APHA) or other governmental regulatory body if outside of the UK. A disease can be listed as notifiable if it is endemic, an example of which is bovine tuberculosis, where the transmission of disease needs to be tracked and controlled. Other notifiable diseases are exotic, for example FMD or bluetongue virus, so that outbreaks can be controlled as soon as possible to limit spread. Currently Johne's disease is not notifiable in Great Britain (Defra & APHA, 2015), however it has been notifiable in Northern Ireland since 1955 (Daera, 2016; Good *et al*., 2009). This difference in situation has arisen due to historical factors which have led to the current regulatory framework in each country. In NI, reports of disease were very sporadic up until the mid 1990's, with only 92 cases been diagnosed between 1932 and 1992 (Good *et al*., 2009). The majority of these cases were found in imported animals, or on farms who had imported animals in the past, so the disease was not thought to be well established and therefore exotic, even though it was widely recognised as an important and wide-spread disease across the rest of Europe. Between 1995

and 2002, 232 JD infections in cattle were reported in NI across 106 herds. This large jump in the number of reports was attributed to the increase in cattle imported from central Europe after the opening of the single European markets (Douarre *et al*., 2010; 85,000 head between 1992 and 2004 compared to 8,383 between 1979 and 1990). The prevalence of JD is still increasing, and has remained as a notifiable disease to allow the authorities to track the spread of JD throughout the country.

Making JD notifiable could be beneficial to the UK due to the high prevalence of disease. This may encourage people to slaughter clinical animals quickly, so reducing the risk of shedding into the rest of the herd, and ensuring that it does not become a welfare issue. To do this effectively there would have to be an improvement in the Se of the detection methods used as part of any control programme, which phage-PCR could possibly provide. This improvement would decrease the number of false-negatives and therefore increase the number of cattle culled and also increase the herd restocking rate. The unforeseen consequence of this could be the spread of other diseases, such as bTB, as was seen after the 2001 FMD outbreak (Carrique-Mas *et al*., 2008; Vial *et al*., 2015). FMD caused in the loss of 6 million animals, with approximately 4.2 million culled for disease control purposes alone. This resulted in widespread restocking of animals and a concomitant resurgence of bovine TB cases (Bourn *et al*., 2002).

The wider reaching effects of improving detection methods with a method such as phage-PCR are the mental health of the farming community. Many are

already under significant stress with low incomes and overall net worth, especially those in the less favoured areas (DEFRA & National Statistics, 2016). Peck *et al*. (2002) used two areas of the UK to investigate how a disease outbreak with significant animal removal, could affect the mental health of farmers. Cumbria, one of the worst hit areas for FMD, was compared to the Scottish Highlands, which remained unaffected during the outbreak in 2001. They found that Cumbrian farmers had a greater risk of psychological morbidity than their Scottish counterparts. This was particular clear on farms that had to deal with a large amount of slaughtered stock as part of the outbreak. It can therefore be understood the effects of the increased loss of cattle caused by the improvement of diagnostics and introduction of notifiable status could have on the mental health of the farming community (Thomas *et al*., 2003). However, it has to be noted that this would only be a short-term effect observed when the improved detection method was initially introduced.

This aside, Khol *et al*. (2007) suggested that a big advantage of declaring clinical JD a notifiable disease with compulsory culling is that the disease is then recognised as important by farmers, veterinarians and consumers. Stakeholder attitudes to JD within the UK are different to what Khol *et al.* (2007) found in Austria. Most UK farmers are very engaged and proactive in control and management of JD, with many partaking in a quarterly testing scheme. This may not be the case if the disease became notifiable with compulsory culling as farmers may not be as willing to test for JD, especially if there was limited compensated for the loss of an animal. A high level of

compensation seems unthinkable for such a prevalent disease at a time when the Government is making financial cutbacks in agricultural sector spending due to exiting the European Union. In turn this may reduce reporting of the disease. The high estimated prevalence of 68 % in the UK (NMR, n.d.), would put too much strain on those that would be involved in the reporting system (veterinary profession and government veterinary officers). The possible strain on reporting systems would be increased if combined with an improvement in detection of MAP-positive animals, as shown here with phage-PCR. However, in countries with an established control programme who are working towards eradication such as Australia, phage-PCR may be able to provide early detection of animals shedding MAP in their faeces, so removing them quickly, reducing the bacterial-load of the herd and limiting transmission, so reaching the point of eradication sooner.

The Government can only facilitate the surveillance of notifiable diseases. There would have to be a demand from across the industry, including other sectors that it affects such as beef and sheep, for JD to be upgraded. A recent example of this is porcine epidemic diarrhoea virus, which became notifiable in 2015 (APHA & Defra, 2015), as there was a call from industry to upgrade its status. On top of this call from industry, the government has to have reason to intervene. There are four reasons that the government would intervene; to protect human health, to protect and promote the welfare of animals, to protect the interests of the wider economy, environment and society, and finally threats to international trade (DEFRA, 2004). The link between MAP and CD could be a reason for the Government to consider upgrading JD to

notifiable. Ellis-Iversen *et al*. (2010) conducted a study of behaviour involving 43 farmers, where all but two believed that they had a social responsibility to produce safe products and control zoonotic disease. Around half also said that the government has a responsibility for safe products, whether this be on the veterinary side, or clean storage and preparation of products before it reaches the consumer, highlighting that this is something that should be tackled on farm and beyond. In the case of JD, this also includes the safe handling of milk past the farm gate, and the improvement of pasteurisation to reduce the risk of zoonosis if the link between JD and CD does become clearer. In this thesis, a survey of retail purchased pasteurised milk was conducted, with samples gained from 14 retailers across Derbyshire, Leicestershire and Nottinghamshire. Viable MAP was found in 10.33 % of pasteurised milk samples, an increase from the 1.8 % found in the last UK wide survey (Grant *et al*., 2002). However, this is not thought to be an increase in prevalence, but an increase in Se of phage-PCR compared to solid culture on HEYM. The survey in pasteurised milk shows that phage-PCR can be versatile in its use, and would provide a simple and rapid method to monitor the amount of MAP surviving the pasteurisation process. It would also be useful to monitor contamination of milk after pasteurisation from the plant.

However, before pasteurisation can be improved, more research into survival of MAP in milk is needed. A possible survival mechanism was also presented in Chapter 6, with MAP found to not be free in the milk, but protected by somatic cells and therefore able to survive pasteurisation. This survival of MAP proves that milk could be a vector for transmission of MAP to humans.

Milk buyers are already trying to get ahead of the possible link between CD and JD by putting clauses into milk contracts to ensure that farmers have a JD control plan in place and are carrying out JD testing on farm, the most popular of which is quarterly milk ab-ELISA. However, some concerns have been raised, both by farmers and veterinarians that this is opening up the doors to producers being penalised by receiving a sub-par price for their product (Action Johne's Conference, pers comm. November 2014). Whilst some will see this as a good incentive to make producers more proactive about JD control, others worry that this will put heavier financial pressure on an already struggling sector, especially as the existing diagnostic tests are not sophisticated enough to effectively control the disease. The farm-gate price of milk is only just recovering after the severe drop in prices starting in 2014, where it reached an average of 20 pence per litre (AHDB Dairy, 2017). To start compulsory testing of all animals with an ineffective test would be a waste of money, possibly pushing some farms out of business. Defra estimate that 14 % of farms have a negative farm business income after paying off all interest, and 3 % of farms have interest payments that are equal or more than their total income, meaning that further borrowing is required to pay the interest charges (Defra, 2014). Hence any additional financial burden could have a serious impact on the number of farms that remain economically viable. Until there is a stronger link between CD and MAP, making the disease a public health risk, it is very unlikely that JD will be made reportable or notifiable due to the effects that it could have on the wider economy.

Although the phage-PCR has proven to detect a group of animals that the other tests (milk ab-ELISA, milk culture and faecal culture) did not, there is still some uncertainty surrounding its validity as an on-farm method. There are differences between the results gained from samples collected in the morning compared to the afternoon, along with increases in the amount of MAP shed during milking. Current milk recording programmes take two samples from each animal, one in the morning and one in the afternoon which are then mixed. Companies are able to do this because of the use of preservatives. Unfortunately, preservatives are not compatible with phage-PCR as they inhibit phage infection, which is why samples were taken from one milking. If phage-PCR were to be widely adopted, care must be taken when designing sampling and testing protocols as consistency will be needed across all herds so that comparisons can be drawn. As suggested in Chapter 5, taking samples during morning milking would be the best option as even though there were less mycobacteria in the milk samples, MAP was more likely to be detected. This could be due to less non-MAP mycobacteria being present, so increasing the probability of selecting a MAP positive plaque for PCR confirmation.

The amount of MAP shed into a cow's milk generally increased during the milking period when samples were taken after every two litres given. This is another factor that would affect the efficiency of detection using phage-PCR. Originally, this was overcome by using the milk sampling equipment for the parlour. However, it was determined that there are non-MAP mycobacteria in the milking machinery, which reduced the ability of phage-PCR to detect MAP as the bacteriophage D29 used within the assay is not MAP-specific. To carry

on using the assay in its current state genetically engineered bacteriophage that are specific to MAP could be used instead of D29.

Unlike ab-ELISA, there would not be a cut-off point that is universally acceptable. A cut-off for phage-PCR would have to be specific to each farm, as Chapters 3 and 4 show that herd-level shedding rates can differ dramatically. A herd with a small number of animals shedding MAP in their milk may opt to remove all those shedding, whereas this would not be viable in a herd where all animals had MAP in their milk. Herds would have to work closely with their routine veterinarian to design a control programme that is suitable to the farm once they received their initial results.

Using blood as a sample matrix rather than milk, as shown by Swift *et al*. (2013), would be an easy change to overcome the difficulties seen using milk. However, this is a difficult sample matrix to obtain as a veterinarian has to be present to take the samples. The use of blood would result in a sterile sample and reduce the issues caused by the presence of other mycobacteria. This means that the current format of the assay could still be used to give accurate enumeration of MAP. For these studies, milk was chosen as the sample matrix due to ease of collection without causing undue stress to the animal or acquiring a Home Office licence. Milk would also work better for commercial purposes as, like the ab-ELISA samples, these could be collected at the same time as the monthly milk recording samples for butterfat, protein and urea. There would then be no increase or adjustments needed for current protocols.

Since the research presented here commenced, the phage-PCR assay has been redeveloped to shorten the time to results to approximately six hours. The time-consuming plating method utilised here has also been removed. The removal of this stage will increase the number of samples that can be processed in one day. In the format used within this research, as one person can only process a maximum of 200 samples in a normal working day, so this would not work under a commercial environment. The new rapid method also removes the 'plaque picking' stage of the method, using the DNA from all of the mycobacteria in the sample, so reducing the risk of missing a MAP cell. However, this does not provide enumeration, which important in those herds who have endemic infection, with most, if not all, cattle shedding MAP. In these situations when using the method used in the research presented here, it would possible to classify animals as not shedding MAP, those shedding low levels of MAP, and those shedding high levels of MAP. The ability to do this will allow the removal of those animals which pose the highest risk to others.

Further investigation into the ability of the phage-PCR assays to predict faecal shedding is needed to clarify and improve the results gained here. The percentage of animals who were shedding MAP into the faeces and detected with phage-PCR before becoming ab-ELISA positive (28 %) is likely an underestimation. This is because there were a number of faecal culture positive samples which were phage positive but MAP-PCR negative, so contained non-specific mycobacteria which reduces the probability of a sample being detected as MAP positive using phage-PCR. It would also be interesting to follow this cohort of cattle forward to see how many other

phage-PCR positive animals become faecal culture positive, and then when or if they become ab-ELISA positive. This would allow further longitudinal comparisons of the diagnostics to see when individual animals would have been removed from the herd with the phage-PCR assay compared to ab-ELISA. A study like this would show the effect the use of phage-PCR may have on the overall MAP-load of the farm, and therefore the speed and success of a control programme. Although, Gao *et al*. (2009) discuss the comparison of milk culture, direct and nested PCR with faecal culture. Here they found that the faecal culture results were not related to any of the three milk-based assays used, so it is interesting to find that phage-PCR may provide some early detection of faecal shedding. When the series of studies presented here were first designed, longitudinal studies on a small number of farms across multiple years without removal of cattle were planned. Here, animals could be followed to determine when they would have been detected as infected with each individual test, and therefore how the tests interact. However, due to the issues discovered with large scale testing, and others mentioned above, this was not possible.

Overall, the sampling techniques used for this piece of research were not suitable for collection of samples for on-farm commercial testing. The suggestions made above would help with its use as a commercial product. However, until the issues discussed are overcome, the use of phage-PCR may be limited to research or use by a veterinarian with input from a research group on a single farm to speed up their control programme. If the issues are addressed, phage-PCR has the potential to be a useful addition to JD control

programmes, as it appears to be more sensitive than other assays currently available or will in the least detect a group of animals that the other tests do not. If used correctly to remove high shedding animals, it could reduce herd level shedding, and therefore the amount of MAP entering the food chain. Even though more research into the assay is needed for use on farm, the assay could readily be used to maintain or improve the cleanliness of pasteurisation plants and to monitor the amount of MAP which enters the food chain.

Whether or not phage-PCR is adopted for use in control programmes, or JD is made notifiable, the disease is a worldwide problem that cannot be controlled by one country's efforts. There is not, and will never be, a one size fits all control plan. This notion has been well accepted at farm-level, so this should this be any different at a national level. Better diagnostic tools to detect an infected animal early and quickly need to be developed, and followed up by correctly advised management practices that suit each individual farm. The control of JD is a long-term goal, but will result in a healthier and more productive national herd. However, this will only be reached if researchers, vets and farmers work together to achieve the common goal.

CHAPTER 8

BIBLIOGRAPHY

AHDB Dairy, 2017. UK, GB AND NI FARMGATE PRICES [WWW Document]. URL https://dairy.ahdb.org.uk/market-information/milk-prices-contracts/farmgate-prices/uk,-gb-and-ni-farmgate-prices/#.WcuGHVLMx-U (accessed 9.27.17).

Aly, S.S., Gardner, I.A., Adaska, J.M., Anderson, R.J., 2015. Off-site rearing of heifers reduces the risk of Mycobacterium avium ssp. paratuberculosis ELISA seroconversion and fecal shedding in a California dairy herd. J. Dairy Sci. 98, 1805–14. doi:10.3168/jds.2014-8759

Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Dennis, M.M., Gould, D.H., Salman, M.D., 2008. Characterization of Mycobacterium avium subspecies paratuberculosis disseminated infection in dairy cattle and its association with antemortem test results. Vet. Microbiol. 127, 300–308. doi:10.1016/j.vetmic.2007.08.017

APHA, 2017. Other Mycobacteria [WWW Document]. URL http://ahvla.defra.gov.uk/External_OV_Instructions/TB_Instructions/Passive_Surveillance/Other_Mycobacteria.html (accessed 2.24.17).

APHA, Defra, 2015. Porcine Epidemic Diarrhoea (PED) becomes a notifiable disease in england [WWW Document]. URL https://www.gov.uk/government/news/porcine-epidemic-diarrhoea-ped-becomes-notifiable-disease-in-england (accessed 1.28.17).

Arla, 2017. Arla Cravendale [WWW Document].

Arsenault, R.J., Maattanen, P., Daigle, J., Potter, A., Griebel, P., Napper, S., 2014. From mouth to macrophage: Mechanisms of innate immune subversion by Mycobacterium avium subsp. Paratuberculosis. Vet. Res. 45, 1–15. doi:10.1186/1297-9716-45-54

Astarie-dequeker, C., N'Diaye, E.-N., Le Cabec, V., Rittig, M.G., Prandi, J., Marideonneau-Parini, I., 1999. The Mannose Receptor Mediates Uptake of Pathogenic and Nonpathogenic Mycobacteria and Bypasses Bactericidal Responses in Human Macrophages. Infect. Immun. 67, 469.

Ayele, W.Y., Svastova, P., Roubal, P., Bartos, M., Pavlik, I., 2005. Mycobacterium avium Subspecies paratuberculosis Cultured from Locally and Commercially Pasteurized Cow ' s Milk in the Czech Republic. Appl. Environ. Microbiol. 71, 1210–1214. doi:10.1128/AEM.71.3.1210

Bach, H., 2015. What Role Does Mycobacterium avium subsp. paratuberculosis Play in Crohn's Disease? Curr. Infect. Dis. Rep. 17, 463. doi:10.1007/s11908-015-0463-z

Bannantine, J.P., Stabel, J.R., 2002. Killing of Mycobacterium avium subspecies paratuberculosis within macrophages. BMC.Microbiol. 2, 2. doi:10.1186/1471-2180-2-2

Barkema, H.W., Hesselink, J.W., McKenna, S.L., Benedictus, G., Groenendaal,

H., 2010. Global Prevalence and Economics of Infection with Mycobacterium avium subsp. paratuberculosis in Ruminants, in: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, pp. 10–21.

Batt, J., Guscetti, F., 2009. Mycobacterium avium supspecies paratuberculosis. pp. 241–243.

Beard, P.M., Rhind, S.M., Buxton, D., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Greig, A., Hutchings, M.R., Stevenson, K., Sharp, J.M., 2001. Natural paratuberculosis infection in rabbits in Scotland. J. Comp. Pathol. 124, 290–9. doi:10.1053/jcpa.2001.0466

Beaver, A., Sweeney, R.W., Hovingh, E., Wolfgang, D.R., Gröhn, Y.T., Schukken, Y.H., 2017. Longitudinal relationship between fecal culture , fecal quantitative PCR , and milk ELISA in Mycobacterium avium ssp . paratuberculosis -infected cows from low-prevalence dairy herds. J. Dairy Sci. 100, 7507–7521. doi:10.3168/jds.2017-12928

Benedictus, a, Mitchell, R.M., Linde-Widmann, M., Sweeney, R., Fyock, T., Schukken, Y.H., Whitlock, R.H., 2008. Transmission parameters of Mycobacterium avium subspecies paratuberculosis infections in a dairy herd going through a control program. Prev. Vet. Med. 83, 215–27. doi:10.1016/j.prevetmed.2007.07.008

Bercovier, H., Vincent, V., Référence, L. De, National, C., Référence, D., 2001. Mycobacterial infections in domestic and wild animals due to Mycobacterium. Rev. Sci. Tech. 20, 265–290.

Berghaus, R., Farver, T., Anderson, R., Jaravata, C., Gardnier, I., 2006. Environmental Sampling for the Detection of Mycobacterium Avium subspecies Paratuberculosis on Large California Dairies. J. Dairy Sci. 89, 963–970.

Blowey, R., Edmondson, P., 2010. Mastitis Control in Dairy Herds, 2nd ed. CAB International, Oxfordshire. doi:10.1017/CBO9781107415324.004

Boelaert, F., Walravens, K., Biront, P., Vermeersch, J.P., Berkvens, D., Godfroid, J., 2000. Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population. Vet. Microbiol. 77, 269–281. doi:10.1016/S0378-1135(00)00312-6

Bolske, G., Herthnek, D., 2010. Diagnosis of Paratuberculosis by PCR, in: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, pp. 267–283.

Botsaris, G., 2010. Development and evaluation of a rapid phage-PCR assay to detect mycobacterium avium subspecies paratuberculosis in dairy products. University of Nottingham.

Botsaris, G., Liapi, M., Kakogiannis, C., Dodd, C.E.R., Rees, C.E.D., 2013.

Detection of Mycobacterium avium subsp. paratuberculosis in bulk tank milk by combined phage-PCR assay: evidence that plaque number is a good predictor of MAP. Int. J. Food Microbiol. 164, 76–80. doi:10.1016/j.ijfoodmicro.2013.03.023

Botsaris, G., Slana, I., Liapi, M., Dodd, C., Economides, C., Rees, C., Pavlik, I., 2010. Rapid detection methods for viable Mycobacterium avium subspecies paratuberculosis in milk and cheese. Int. J. Food Microbiol. 141, S87-90. doi:10.1016/j.ijfoodmicro.2010.03.016

Botsaris, G., Swift, B.M.C., Slana, I., Liapi, M., Christodoulou, M., Hatzitofi, M., Christodoulou, V., Rees, C.E.D., 2016. Detection of viable Mycobacterium avium subspecies paratuberculosis in powdered infant formula by phage-PCR and confirmed by culture. Int. J. Food Microbiol. 216, 91–94. doi:10.1016/j.ijfoodmicro.2015.09.011

Bourn, J., Eales, R., Thomas, P., Bostock, D., Lingard, S., Derbyshire, I., Burmiston, A., Kitson, H., 2002. The 2001 Outbreak of Foot and Mouth Disease, National Audit Office.

Boutinaud, M., Jammes, H., 2002. Potential uses of milk epithelial cells: a review. Reprod. Nutr. Dev. 42, 133–147. doi:10.1051/rnd

Bradley, A.J., 2002. Bovine mastitis: An evolving disease. Vet. J. 164, 116–128. doi:10.1053/tvjl.2002.0724

Bradner, L., Robbe-Austerman, S., Beitz, D.C., Stabel, J.R., 2013. Chemical Decontamination with N-Acetyl-L-Cysteine-Sodium Hydroxide Improves Recovery of Viable Mycobacterium avium subsp. paratuberculosis Organisms from Cultured Milk. J. Clin. Microbiol. 51, 2139–2146. doi:10.1128/JCM.00508-13

Bull, T.J., Mcminn, E.J., Sidi-boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R., Hermon-taylor, J., 2003. Detection and Verification of Mycobacterium avium subsp . paratuberculosis in Fresh Ileocolonic Mucosal Biopsy Specimens from Individuals with and without Crohn ' s Disease Detection and Verification of Mycobacterium avium subsp . paratuberculosis in Fres. J. Clin. Microbiol. 41, 2915–2923. doi:10.1128/JCM.41.7.2915

Caldow, G., Gunn, G.J., 2002. Assessment of surveillance and control of Johne ' s disease in farm animals in GB.

Carrique-Mas, J.J., Medley, G.F., Green, L.E., 2008. Risks for bovine tuberculosis in British cattle farms restocked after the foot and mouth disease epidemic of 2001. Prev. Vet. Med. 84, 85–93. doi:10.1016/j.prevetmed.2007.11.001

Cetinkaya, B., Erdogan, H.M., Morgan, K.L., 1997. Relationships between the presence of Johne's disease and farm and management factors in dairy

cattle in England. Prev. Vet. Med. 32, 253–266. doi:10.1016/S0167-5877(97)00028-7

Chacon, O., Bermudez, L.E., Barletta, R.G., 2004. Johne's disease, inflammatory bowel disease, and Mycobacterium paratuberculosis. Annu. Rev. Microbiol. 58, 329–63. doi:10.1146/annurev.micro.58.030603.123726

CHeCS, 2017. About Us [WWW Document]. URL http://www.checs.co.uk/about-us/ (accessed 2.25.17).

Chi, J., VanLeeuwen, J. a., Weersink, A., Keefe, G.P., 2002. Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, Mycobacterium avium subspecies paratuberculosis, and Neospora caninum. Prev. Vet. Med. 55, 137–153. doi:10.1016/S0167-5877(02)00094-6

Chiodini, R.J., 1996. Immunology: Resistance to Paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12, 313–343. doi:10.1016/S0749-0720(15)30409-6

Chiodini, R.J., Hermon-Taylor, J., 1993. The thermal resistance of Mycobacterium paratuberculosis in raw milk under conditions simulating pasteurization. J. Vet. Diagn. Invest. 5, 629–31.

Coad, M., Clifford, D., Vordermeier, H., Whelan, A., 2013. The consequences of vaccination with the Johne's disease vaccine, Gudair, on diagnosis of bovine tuberculosis. Vet. Rec. 172.

Collins, M.T., Morgan, I.R., 1991. Epidemiological model of paratuberculosis in dairy cattle. Prev. Vet. Med. 11, 131–146. doi:10.1016/S0167-5877(05)80035-2

Collins, M.T., Wells, S.J., Petrini, K.R., Collins, J.E., Schultz, R.D., Whitlock, R.H., 2005. Evaluation of Five Antibody Detection Tests for Diagnosis of Bovine Paratuberculosis. Clin. Diagn. Lab. Immunol. 12, 685–692. doi:10.1128/CDLI.12.6.685

Cook, K.L., Britt, J.S., Bolster, C.H., 2010. Survival of Mycobacterium avium subsp. paratuberculosis in biofilms on livestock watering trough materials. Vet. Microbiol. 141, 103–109. doi:10.1016/j.vetmic.2009.08.013

Corner, L.A., Pearson, C.W., 1979. RESPONSE OF CATTLE TO INOCULATION WITH ATYPICAL MYCOBACTERIA ISOLATED FROM SOIL. Aust. Vet. J. 55, 6–9.

Corti, S., Stephan, R., 2002. Detection of Mycobacterium avium subspecies paratuberculosis specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. BMC Microbiol. 2, 15. doi:10.1186/1471-2180-2-15

Cousins, D. V, Whittington, R., Marsh, I., Masters, A., Evans, R.J., Kluver, P., 1999. Mycobacteria distenct from Mycobacterium avium subsp. paratuberculosis isolated from the faeces of ruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis. Mol. Cell. Probes 13, 431–42. doi:10.1006/mcpr.1999.0275

Coussens, P.M., 2001. Mycobacterium paratuberculosis and the bovine immune system. Anim. Heal. Res. Reveiws 2, 141–162. doi:10.1079/AHRR200134

Coussens, P.M., Lamont, E.A., Kabara, E., Sreevatsan, S., 2010. Host-Pathogen Interactions and Intracellular Survival of Mycobacterium avium subsp. paratuberculosis, in: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, pp. 109–125.

Covert, T.C., Rodgers, M.R., Reyes, A.L., Jr, G.N.S., Stelma, G.N., 1999. Occurrence of Nontuberculous Mycobacteria in Environmental Samples Occurrence of Nontuberculous Mycobacteria in Environmental Samples. Appl. Environ. Microbiol. 65, 2492–2496.

Daera, 2016. Notifiable diseases in Northern Ireland [WWW Document]. URL https://www.daera-ni.gov.uk/articles/notifiable-diseases-northern-ireland (accessed 1.1.17).

Dailloux, M., Laurain, C., Weber, M., Hartemann, P., 1999. Water and Nontuberculous Mycobacteria. Water Res. 33, 2219–2228. doi:21.05.15

Dairy Co, 2011. Farmer Water Survey Report. Warwickshire.

Daniels, M.J., Henderson, D., Greig, a, Stevenson, K., Sharp, J.M., Hutchings, M.R., 2003. The potential role of wild rabbits Oryctolagus cuniculus in the epidemiology of paratuberculosis in domestic ruminants. Epidemiol. Infect. 130, 553–9.

Davidson, R.S., Marion, G., White, P.C.L., Hutchings, M.R., 2009. Use of host population reduction to control wildlife infection: rabbits and paratuberculosis. Epidemiol. Infect. 137, 131–138. doi:10.1017/S0950268808000642

Davidson, R.S., McKendrick, I.J., Wood, J.C., Marion, G., Greig, A., Stevenson, K., Sharp, M., Hutchings, M.R., 2012. Accounting for uncertainty in model-based prevalence estimation: paratuberculosis control in dairy herds. BMC Vet. Res. 8, 159.

Defra, 2016. TB strategy ahead of schedule as England set to apply for officially TB-free status for half the country [WWW Document]. Bov. Tuberc. URL https://www.gov.uk/government/news/tb-strategy-ahead-of-schedule-as-england-set-to-apply-for-officially-tb-free-status-for-half-the-country (accessed 2.27.17).

Defra, 2014. Balance sheet analysis and farming performance , England

2015/16. London.

DEFRA, 2004. Animal Health and Welfare Strategy for Great Britain.

Defra, APHA, 2015. Notifiable diseases in animals [WWW Document]. URL https://www.gov.uk/government/collections/notifiable-diseases-in-animals (accessed 1.1.16).

DEFRA, National Statistics, 2016. Farm Accounts in England – Results from the Farm Business Survey 2015/16, Farm Business Survey.

Dohoo, I.R., Meek, A.H., 1982. Somatic cell counts in bovine milk. Can. Vet. journal. La Rev. vétérinaire Can. 23, 119–25.

Donat, K., Kube, J., Dressel, J., Einax, E., Pfeffer, M., 2017. Detection of Mycobacterium avium subspecies paratuberculosis in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture 975–985. doi:10.1017/S0950268814002465

Dong, F., 2006. The outlook for Asian dairy markets: The role of demographics, income, and prices. Food Policy 31, 260–271. doi:10.1016/j.foodpol.2006.02.007

Douarre, P.E., Cashman, W., Buckley, J., Coffey, A., O'Mahony, J.M., 2010. Isolation and detection of Mycobacterium avium subsp. paratuberculosis (MAP) from cattle in Ireland using both traditional culture and molecular based methods. Gut Pathog. 2, 11. doi:10.1186/1757-4749-2-11

Dundee, L., Grant, I.R., Ball, H.J., Rowe, M.T., 2001. Comparative evaluation of four decontamination protocols for the isolation of Mycobacterium avium subsp. paratuberculosis from milk. Lett. Appl. Microbiol. 33, 173–7.

Dunn, B.L., Hodgson, D.J., 1982. ' Atypical ' mycobacteria in milk. J. Appl. Bacteriol. 52, 373–376.

Eamens, G.J., Whittington, R.J., Marsh, I.B., Turner, M.J., Saunders, V., Kemsley, P.D., Rayward, D., 2000. Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. Vet. Microbiol. 77, 357–367. doi:10.1016/S0378-1135(00)00321-7

Eisenberg, S.W.F., Veldman, E., Rutten, V.P.M.G., Koets, A.P., 2015. A longitudinal study of factors influencing the result of a Mycobacterium avium ssp. paratuberculosis antibody ELISA in milk of dairy cows. J. Dairy Sci. 98, 2345–55. doi:10.3168/jds.2014-8380

Elizondo-Salazar, J.A., Jones, C.M., Heinrichs, A.J., 2010. Evaluation of calf milk pasteurization systems on 6 Pennsylvania dairy farms. J. Dairy Sci. 93, 5509–13. doi:10.3168/jds.2010-3460

Ellingson, J.L.E., Anderson, J.L., Koziczkowski, J.J., Radcliff, R.P., Sloan, S.J., Allen, S.E., Sullivan, N.M., 2005. Detection of viable Mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR. J. Food Prot. 68, 966–972.

Elliott, G.N., Hough, R.L., Avery, L.M., Maltin, C. a, Campbell, C.D., 2014. Environmental risk factors in the incidence of Johne's disease. Crit. Rev. Microbiol. 7828, 1–20. doi:10.3109/1040841X.2013.867830

Ellis-Iversen, J., Cook, A.J.C., Watson, E., Nielen, M., Larkin, L., Wooldridge, M., Hogeveen, H., 2010. Perceptions, circumstances and motivators that influence implementation of zoonotic control programs on cattle farms. Prev. Vet. Med. 93, 276–285. doi:10.1016/j.prevetmed.2009.11.005

Emery, D., Whittington, R., 2004. An evaluation of mycophage therapy, chemotherapy and vaccination for control of subsp. infection. Vet. Microbiol. 104, 143–155. doi:10.1016/j.vetmic.2004.08.014

Englund, S., Bolske, G., Johansson, K.E., 2002. An IS900-like sequence found in a Mycobacterium sp. other than Mycobacterium avium subsp. paratuberculosis. FEMS Microbiol Lett 209, 267–271.

Espejo, L.A., Kubat, N., Godden, S.M., Wells, S.J., 2013. Effect of delayed exposure of cattle to Mycobacterium avium subsp paratuberculosis on the development of subclinical and clinical Johne's disease. Am. J. Vet. Res. 74, 1304–1310.

Espejo, L.A., Zagmutt, F.J., Groenendaal, H., Muñoz-Zanzi, C., Wells, S.J., 2015. Evaluation of performance of bacterial culture of feces and serum ELISA across stages of Johne's disease in cattle using a Bayesian latent class model. J. Dairy Sci. 98, 8227–8239. doi:10.3168/jds.2014-8440

Falkinham, J.O., Norton, C.D., Mark, W., 2001. Factors Influencing Numbers of Mycobacterium avium , Mycobacterium intracellulare , and Other Mycobacteria in Drinking Water Distribution Systems Factors Influencing Numbers of Mycobacterium avium , Mycobacterium intracellulare , and Other Mycobacteria in. Appl. Environ. Microbiol. 67, 1225–1231. doi:10.1128/AEM.67.3.1225

FDA, n.d. Powdered Infant Formula: An Overview of Manufacturing Processes [WWW Document]. URL https://www.fda.gov/ohrms/dockets/ac/03/briefing/3939b1_tab4b.htm

Fecteau, M.-E., Whitlock, R.H., 2010. Paratuberculosis in Cattle, in: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, pp. 144–156.

Fecteau, M.E., Whitlock, R.H., 2011. Treatment and Chemoprophylaxis for Paratuberculosis. Vet. Clin. North Am. - Food Anim. Pract. 27, 547–557. doi:10.1016/j.cvfa.2011.07.002

Fecteau, M.E., Whitlock, R.H., 2010. Paratuberculosis in Cattle, in: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, pp. 144–156.

Feller, M., Huwiler, K., Stephan, R., Altpeter, E., Shang, a, Furrer, H., Pfyffer, G.E., Jemmi, T., Baumgartner, a, Egger, M., 2007. Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. Lancet Infect. Dis. 7, 607–613. doi:10.1016/S1473-3099(07)70211-6

Flesch, I., Kaufmann, S., 1994. Role of macrophages and αβ T lymphocytes in early interleukin 10 production during Listeria monocytogenes infection. Int. Immunol. 6, 463–468.

Forsbäck, L., Lindmark-Månsson, H., Andrén, a, Akerstedt, M., Andrée, L., Svennersten-Sjaunja, K., 2010. Day-to-day variation in milk yield and milk composition at the udder-quarter level. J. Dairy Sci. 93, 3569–3577. doi:10.3168/jds.2009-3015

Franco, M.M.J., Paes, A.C., Ribeiro, M.G., de Figueiredo Pantoja, J.C., Santos, A.C.B., Miyata, M., Leite, C.Q.F., Motta, R.G., Listoni, F.J.P., 2013. Occurrence of mycobacteria in bovine milk samples from both individual and collective bulk tanks at farms and informal markets in the southeast region of Sao Paulo, Brazil. BMC Vet. Res. 9, 85. doi:10.1186/1746-6148-9-85

Gao, A., Odumeru, J., Raymond, M., Hendrick, S., Duffield, T., Mutharia, L., 2009. Comparison of milk culture, direct and nested polymerase chain reaction (PCR) with fecal culture based on samples from dairy herds infected with Mycobacterium avium subsp. paratuberculosis. Can. J. Vet. Res. 73, 58–64.

Gao, a, Mutharia, L., Chen, S., Rahn, K., Odumeru, J., 2002. Effect of pasteurization on survival of Mycobacterium paratuberculosis in milk. J. Dairy Sci. 85, 3198–3205. doi:10.3168/jds.S0022-0302(02)74408-1

Garcia, A.B., Shalloo, L., 2015. Invited review: The economic impact and control of paratuberculosis in cattle. J. Dairy Sci. 98, 5019–5039. doi:10.3168/jds.2014-9241

Geraghty, T., Graham, D. a., Mullowney, P., More, S.J., 2014. A review of bovine Johne's disease control activities in 6 endemically infected countries. Prev. Vet. Med. 116, 1–11. doi:10.1016/j.prevetmed.2014.06.003

Gilmour, N.J.L., Nisbet, D.I., Brotherson, J.G., 1965. Experimental Oral Infection of Calves. J. Comp. Pathol. 75, 281. doi:10.1016/0021-9975(65)90033-2

Godden, S.M., Wells, S., Donahue, M., Stabel, J., Oakes, J.M., Sreevatsan, S.,

Fetrow, J., 2015. Effect of feeding heat-treated colostrum on risk for infection with Mycobacterium avium ssp. paratuberculosis, milk production, and longevity in Holstein dairy cows. J. Dairy Sci. 98, 5630–5641. doi:10.3168/jds.2015-9443

Goh, K.L., Xiao, S.D., 2009. Inflammatory bowel disease: A survey of the epidemiology in Asia. J. Dig. Dis. 10, 1–6. doi:10.1111/j.1751-2980.2008.00355.x

Good, M., Clegg, T., Sheridan, H., Yearsely, D., O'Brien, T., Egan, J., Mullowney, P., 2009. Prevalence and distribution of paratuberculosis (Johne's disease) in cattle herds in Ireland. Ir. Vet. J. 62, 597–606. doi:10.1186/2046-0481-62-9-597

Grant, I.R., Ball, H.J., Neill, S.D., Rowe, M.T., Grant, I.R., Ball, H.J., Neill, S.D., 1996a. Inactivation of Mycobacterium paratuberculosis in cows ' milk at pasteurization temperatures. Appl. Environ. Microbiol. 62, 631–636.

Grant, I.R., Ball, H.J., Neill, S.D., Rowe, M.T., Grant, I.R., Ball, H.J., Neill, S.D., 1996b. Inactivation of Mycobacterium paratuberculosis in cows ' milk at pasteurization temperatures . These include : Inactivation of Mycobacterium paratuberculosis in Cows ' Milk at Pasteurization Temperatures 62, 631–636.

Grant, I.R., Ball, H.J., Rowe, M.T., 2002. Incidence of Mycobacterium paratuberculosis in Bulk Raw and Commercially Pasteurized Cows ' Milk from Approved Dairy Processing Establishments in the United Kingdom Incidence of Mycobacterium paratuberculosis in Bulk Raw and Commercially Pasteurized Cows . Appl. Environ. Microbiol. 68, 2428–2435. doi:10.1128/AEM.68.5.2428

Grant, I.R., Ball, H.J., Rowe, M.T., 1998. Effect of high-temperature, short-time (HTST) pasteurization on milk containing low numbers of Mycobacterium paratuberculosis. Lett. Appl. Microbiol. 26, 166–70.

Grant, I.R., Ball, H.J., Rowe, M.T., 1996c. Thermal inactivation of several Mycobacterium spp. in milk by pasteurization. Lett. Appl. Microbiol. 22, 253–256.

Grant, I.R., Pope, C.M., O'Riordan, L.M., Ball, H.J., Rowe, M.T., 2000. Improved detection of Mycobacterium avium subsp. paratuberculosis In milk by immunomagnetic PCR. Vet. Microbiol. 77, 369–78.

Grant, I.R., Rowe, M.T., 2004. Effect of chemical decontamination and refrigerated storage on the isolation of Mycobacterium avium subsp. paratuberculosis from heat-treated milk. Lett. Appl. Microbiol. 38, 283–288. doi:10.1111/j.1472-765X.2004.01498.x

Grant, I.R., Williams, A.G., Rowe, M.T., Muir, D.D., 2005. Efficacy of various pasteurization time-temperature conditions in combination with

homogenization on inactivation of Mycobacterium avium subsp. paratuberculosis in milk. Appl. Environ. Microbiol. 71, 2853–2861. doi:10.1128/AEM.71.6.2853-2861.2005

Greenstein, R.J., 2003. Personal view Is Crohn ' s disease caused by a mycobacterium ? Comparisons with leprosy , tuberculosis , and Johne ' s disease Personal view. Lancet Infect. Dis. 3, 507–514.

Groenendaal, H., Nielen, M., Jalvingh, A.W., Horst, S.H., Galligan, D.T., Hesselink, J.W., 2002. A simulation of Johne's disease control. Prev. Vet. Med. 54, 225–245. doi:10.1016/S0167-5877(02)00027-2

Groenendaal, H., Zagmutt, F.J., Patton, E. a., Wells, S.J., 2015. Cost-benefit analysis of vaccination against Mycobacterium avium ssp. paratuberculosis in dairy cattle, given its cross-reactivity with tuberculosis tests. J. Dairy Sci. 98, 1–15. doi:10.3168/jds.2014-8914

Hall-Stoodley, L., Lappin-Scott, H., 1998. Biofilm formation by the rabidly growing mycobacterial species Mycobacterium fortuitum . FEMS Microbiol.Lett. 168, 77–84.

Hanks, J.D., Taylor, N.M., Kossaibati, M.A., 2014. Is targeted milk sampling an effective means of detecting Johne ' s disease in dairy herds ? Cattle Pract. 22, 26–34.

Harris, B.N., Barletta, R.G., 2001. Mycobacterium avium subsp . paratuberculosis in Veterinary Medicine. Clin. Microbiol. Reveiws 14, 489–512. doi:10.1128/CMR.14.3.489

Holsinger, V.H., Rajkowski, K.T., Stabel, J.R., 1997. Milk pasteurisation and safety: a brief history and update. Rev. Sci. Tech. 16, 441–451.

Jacobs, M., Brown, N., Allie, N., Gulert, R., Ryffel, B., 2000. Increased resistance to mycobacterial infection in the absence of interleukin-10. Immunology 100, 494–501.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Rev Sci Tech 17, 469–526.

James, D., 2017. Milk buyers ditch farmers not tackling Johne's disease [WWW Document]. Farmers Wkly. URL http://www.fwi.co.uk/livestock/milk-buyers-ditch-farmers-not-tackling-johnes-disease.htm (accessed 2.24.17).

Jørgensen, J.B., 1982. An improved medium for culture of Mycobacterium paratuberculosis from bovine faeces. Acta Vet Scand 23, 325–35.

Judge, J., Greig, A., Kyriazakis, I., Hutchings, M.R., 2005. Ingestion of faeces by grazing herbivores—risk of inter-species disease transmission. Agric. Ecosyst. Environ. 107, 267–274. doi:10.1016/j.agee.2004.10.028

Jungersen, G., Huda, A., Hansen, J.J., Lind, P., 2002. Interpretation of the gamma interferon test for diagnosis of subclinical paratuberculosis in cattle. Clin. Diagn. Lab. Immunol. 9, 453–460. doi:10.1128/CDLI.9.2.453

Kang, T.J., Chae, G.T., 2001. Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. FEMS Immunol. Med. Microbiol. 31, 53–58. doi:10.1016/S0928-8244(01)00239-5

Karreman, H., n.d. Johnes Disease [WWW Document]. Northeast Org. Dairy Prod. URL http://www.nodpa.com/production_johnes_disease-11-10-09.shtml (accessed 8.16.17).

Kawaji, S., Nagata, R., Mori, Y., 2014. Detection and Confirmation of Mycobacterium avium subsp. paratuberculosis in Direct Quantitative PCR Positive Fecal Samples by the Manual Fluorescent MGIT Culture System. J. Vet. Med. Sci. 76, 65–72. doi:10.1292/jvms.13-0366

Kazwala, R.R., Daborn, C.J., Kusiluka, L.J.M., Jiwa, S.F.H., Sharp, J.M., Kambarage, D.M., 1998. Isolation of Mycobacterium Species From Raw Milk of Pastoral Cattle of the Southern Highlands of Tanzania. Trop. Anim. Heal. Prod. 30, 233–239.

Kennedy, A.E., da Silva, A.T., Byrne, N., Govender, R., MacSharry, J., O'Mahony, J., Sayers, R.G., 2014. The single intradermal cervical comparative test interferes with Johne's disease ELISA diagnostics. Front. Immunol. 5, 1–8. doi:10.3389/fimmu.2014.00564

Kennedy, B.W., Sethar, M.S., Tong, a. K.W., Moxley, J.E., Downey, B.R., 1982. Environmental Factors Influencing Test-Day Somatic Cell Counts in Holsteins. J. Dairy Sci. 65, 275–280. doi:10.3168/jds.S0022-0302(82)82188-7

Khalifeh, M.S., Stabel, J.R., 2004. Effects of Gamma Interferon , Interleukin-10 , and Transforming Growth Factor β on the Survival of Mycobacterium avium subsp . paratuberculosis in Monocyte-Derived Macrophages from Naturally Infected Cattle. Infect. Immun. 72, 1974–1982. doi:10.1128/IAI.72.4.1974

Khol, J.L., Damoser, J., Dünser, M., Baumgartner, W., 2007. Paratuberculosis, a notifiable disease in Austria-Current status, compulsory measures and first experiences. Prev. Vet. Med. 82, 302–307. doi:10.1016/j.prevetmed.2007.06.002

King, C.H., Shotts, E.B., Wooley, R.E., Porter, K.G., 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl. Environ. Microbiol. 54, 3023–3033. doi:file://Z:\References\Text Files\00000003955.txt

Kirkpatrick, B.W., Shook, G.E., 2011. Genetic Susceptibility to Paratuberculosis. Vet. Clin. North Am. - Food Anim. Pract. 27, 559–571.

doi:10.1016/j.cvfa.2011.07.003

Klinkenberg, D., Koets, A., 2015. The long subclinical phase of Mycobacterium avium ssp. paratuberculosis infections explained without adaptive immunity. Vet. Res. 46, 63. doi:10.1186/s13567-015-0202-3

Koenig, G., Hoffsis, G., Shulaw, W., Bech-Nielsen, S., Rings, D., St-Jean, G., 1993. Isolation of Mycobacterium paratuberculosis from mononuclear cells in tissues, blood, and mammary glands of cows with advanced paratuberculosis. Am. J. Vet. Res. 54, 1441–1445.

Koets, A., Santema, W., Mertens, H., Oostenrijk, D., Keestra, M., Overdijk, M., Labouriau, R., Franken, P., Frijters, A., Nielen, M., Rutten, V., 2010. Susceptibility to paratuberculosis infection in cattle is associated with single nucleotide polymorphisms in Toll-like receptor 2 which modulate immune responses against Mycobacterium avium subspecies paratuberculosis. Prev. Vet. Med. 93, 305–315. doi:10.1016/j.prevetmed.2009.11.008

Koets, A.P., Eda, S., Sreevatsan, S., 2015. The within host dynamics of Mycobacterium avium ssp. paratuberculosis infection in cattle: where time and place matter. Vet. Res. 46, 61. doi:10.1186/s13567-015-0185-0

Koets, a. P., Adugna, G., Janss, L.L.G., van Weering, H.J., Kalis, C.H.J., Wentink, G.H., Rutten, V.P.M.G., Schukken, Y.H., 2000. Genetic Variation of Susceptibility to Mycobacterium avium subsp. paratuberculosis Infection in Dairy Cattle. J. Dairy Sci. 83, 2702–2708. doi:10.3168/jds.S0022-0302(00)75164-2

Kralik, P., Pribylova-Dziedzinska, R., Kralova, A., Kovarcik, K., Slana, I., 2014. Evidence of passive faecal shedding of Mycobacterium avium subsp. paratuberculosis in a Limousin cattle herd. Vet. J. 201, 91–94. doi:10.1016/j.tvjl.2014.02.011

Kudahl, A.B., Østergaard, S., Sørensen, J.T., Nielsen, S.S., 2007a. A stochastic model simulating paratuberculosis in a dairy herd. Prev. Vet. Med. 78, 97–117. doi:10.1016/j.prevetmed.2006.05.015

Kudahl, A.B., Sørensen, J.T., Nielsen, S.S., Østergaard, S., 2007b. Simulated economic effects of improving the sensitivity of a diagnostic test in paratuberculosis control. Prev. Vet. Med. 78, 118–129. doi:10.1016/j.prevetmed.2006.10.004

Kuroda, K., Brown, E.J., Telle, W.B., Russell, D.G., Ratliff, T.L., 1993. Characterization of the internalization of bacillus Calmette-Guerin by human bladder tumor cells. J. Clin. Invest. 91, 69–76. doi:10.1172/JCI116202

Laurin, E.L., 2015. Study of shedding patterns of Mycobacterium avium subspecies paratuberculosis in feces, milk, and colostrum of dairy cows

and the development of novel early detection methods for Johne's disease. University of Prince Edward Island.

Laurin, E.L., Chaffer, M., McClure, J.T., McKenna, S.L.B., Keefe, G.P., 2015. The association of detection method, season, and lactation stage on identification of fecal shedding in Mycobacterium avium ssp. paratuberculosis infectious dairy cows. J. Dairy Sci. 98, 211–20. doi:10.3168/jds.2014-8406

Leach, K.A., Archer, S.C., Breen, J.E., Green, M.J., Ohnstad, I.C., Tuer, S., Bradley, A.J., 2015. Recycling manure as cow bedding: Potential benefits and risks for UK dairy farms. Vet. J. doi:10.1016/j.tvjl.2015.08.013

Le Dantec, C., Duguet, J.P., Montiel, A., Dumoutier, N., Dubrou, S., Vincent, V., 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. 68, 5318–5325. doi:10.1128/AEM.68.11.5318

Leitner, G., Chaffer, M., Krifucks, O., Glickman, A., Ezra, F., Saran, A., 2000a. Milk leucocyte populations in heifers free of udder infection. J. Vet. Med. Ser. B 47, 133–138. doi:10.1046/j.1439-0450.2000.00329.x

Leitner, G., Shoshani, E., Krifucks, O., Chaffer, M., Saran, A., 2000b. Milk leucocyte population patterns in bovine udder infection of different aetiology. J. Vet. Med. Ser. B 47, 581–589. doi:10.1046/j.1439-0450.2000.00388.x

Lindmark-Mansson, H., Branning, C., Alden, G., Paulsson, M., 2006. Relationship between somatic cell count, individual leukocyte populations and milk components in bovine udder quarter milk. Int. Dairy J. 16, 717–727. doi:10.1016/j.idairyj.2005.07.003

Linn, J.G., 1988. Factors Affecting the Composition of Milk from Dairy Cows, in: Designing Foods: Animal Product Options in the Marketplace. National Academy Press, Washington D.C., p. 224. doi:10.17226/661

Logar, K., Kopinč, R., Bandelj, P., Starič, J., Lapanje, A., Ocepek, M., 2012. Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of Mycobacterium avium subsp. paratuberculosis in subclinically infected dairy cattle: comparison with faecal culture, milk real-time PCR and milk ELISA. BMC Vet. Res. 8, 49. doi:10.1186/1746-6148-8-49

Lombard, J.E., 2011. Epidemiology and Economics of Paratuberculosis. Vet. Clin. North Am. - Food Anim. Pract. 27, 525–535. doi:10.1016/j.cvfa.2011.07.012

Lombard, J.E., Wagner, B.A., Smith, R.L., Mccluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B., Salman, M.D., 2006. Evaluation of Environmental Sampling and Culture to Determine Mycobacterium avium subspecies

paratuberculosis Distribution and Herd Infection Status on US Dairy Operations. J. Dairy Sci. 89, 4163–4171. doi:10.3168/jds.S0022-0302(06)72461-4

Løvendahl, P., Bjerring, M. a, 2006. Detection of carryover in automated milk sampling equipment. J. Dairy Sci. 89, 3645–3652. doi:10.3168/jds.S0022-0302(06)72404-3

Lu, Z., Mitchell, R.M., Smith, R.L., Van Kessel, J.S., Chapagain, P.P., Schukken, Y.H., Grohn, Y.T., 2008. The importance of culling in Johne's disease control. J. Theor. Biol. 254, 135–46. doi:10.1016/j.jtbi.2008.05.008

Lu, Z., Schukken, Y.H., Smith, R.L., Grohn, Y.T., 2010. Stochastic simulations of a multi-group compartmental model for Johne's disease on US dairy herds with test-based culling intervention. J. Theor. Biol. 264, 1190–201. doi:10.1016/j.jtbi.2010.03.034

Lund, B.M., Gould, G.W., Rampling, A.M., 2002. Pasteurization of milk and the heat resistance of Mycobacterium avium subsp. paratuberculosis: A critical review of the data. Int. J. Food Microbiol. 77, 135–145. doi:10.1016/S0168-1605(02)00057-0

Lynch, D., Jordan, K.N., Kelly, P.M., Freyne, T., Murphy, P.M., 2007. Heat sensitivity of Mycobacterium avium ssp. paratuberculosis in milk under pilot plant pasteurization conditions. Int. J. Dairy Technol. 60, 98–104. doi:10.1111/j.1471-0307.2007.00317.x

Magombedze, G., Ngonghala, C.N., Lanzas, C., 2013. Evalution of the "Iceberg Phenomenon" in Johne's Disease through Mathematical Modelling. PLoS One 8. doi:10.1371/journal.pone.0076636

Marcé, C., Ezanno, P., Seegers, H., Pfeiffer, D.U., Fourichon, C., 2011. Within-herd contact structure and transmission of Mycobacterium avium subspecies paratuberculosis in a persistently infected dairy cattle herd. Prev. Vet. Med. 100, 116–125. doi:10.1016/j.prevetmed.2011.02.004

McAloon, C.G., Doherty, M.L., Whyte, P., O'Grady, L., More, S.J., Messam, L.L.M., Good, M., Mullowney, P., Strain, S., Green, M.J., 2016. Bayesian estimation of prevalence of paratuberculosis in dairy herds enrolled in a voluntary Johne's Disease Control Programme in Ireland. Prev. Vet. Med. 128, 95–100. doi:10.1016/j.prevetmed.2016.04.014

McDonald, W., O'Riley, K., Schroen, C., Condron, R.J., 2005. Heat inactivation of Mycobacterium avium subsp. paratuberculosis in milk. Appl. Environ. Microbiol. 71, 1785–1789. doi:10.1128/AEM.71.4.1785

McDonald, W.L., Ridge, S.E., Hope, A.F., Condron, R.J., 1999. Evaluation of diagnostic tests for Johne's disease in young cattle. Aust. Vet. J. 77, 113–119. doi:10.1111/j.1751-0813.1999.tb11679.x

McKenna, S.L.B., Keefe, G.P., Barkema, H.W., Sockett, D.C., 2005. Evaluation

of three ELISAs for Mycobacterium avium subsp. paratuberculosis using tissue and fecal culture as comparison standards. Vet. Microbiol. 110, 105–11. doi:10.1016/j.vetmic.2005.07.010

Meadows, R., 2004. Preventing Johne's disease is good all-around dairy practice. Calif. Agric. 58, 137.

Means, T.K., Wang, S., Lien, E., Yoshimura, a, Golenbock, D.T., Fenton, M.J., 1999. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J. Immunol. 163, 3920–7.

Mee, J.F., Richardson, E., 2008. Epidemiology and economic impact of Johne ' s disease in Irish dairy herds Epidemiology and economic impact of Johne ' s disease in Irish dairy herds.

Menard, L., Vanasse, C., Diaz, C., Rivar, G., 1983. Mycobacterium chelonei mastitis in a Quebec dairy herd. Can. Vet. J. 24, 305–307.

Merkal, R.S., Larsen, A.B., Booth, G.D., 1975. Analysis of the effcet of inapparent bovine paratuberculosis. Am. J. Vet. Res. 36, 837–838.

Merkal, R.S., Miller, J.M., Hintz, A.M., Bryner, J.H., 1982. Intrauterine inoculation of Mycobacterium paratuberculosis into guinea pigs and cattle. Am. J. Vet. Res. 43, 676–678.

Mihajlovic, B., Klassen, M., Springthorpe, S., Couture, H., Farber, J., 2011. Assessment of Sources of Exposure for Mycobacterium avium subsp. paratuberculosis in Food and Water. Int. Food Risk Anal. J. 1, 1. doi:10.5772/10681

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T., Hermon-Taylor, J., 1996. IS900 PCR to detect Mycobacterium paratuberculosis in retail supplies of whole pasteurized cows' milk in England and Wales. Appl. Environ. Microbiol. 62, 3446–3452.

Mitchell, R.M., Schukken, Y., Koets, A., Weber, M., Bakker, D., Stabel, J., Whitlock, R.H., Louzoun, Y., 2015. Differences in intermittent and continuous fecal shedding patterns between natural and experimental Mycobacterium avium subspecies paratuberculosis infections in cattle. Vet. Res. 46, 66. doi:10.1186/s13567-015-0188-x

Mole, R., Trollip, A., Abrahams, C., Bosman, M., Albert, H., 2007. Improved contamination control for a rapid phage-based rifampicin resistance test for Mycobacterium tuberculosis. J. Med. Microbiol. 56, 1334–9. doi:10.1099/jmm.0.46936-0

Momotani, E., Whipple, D.L., Thiermann, a B., Cheville, N.F., 1988. Role of M cells and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. Vet. Pathol. 25, 131–137. doi:10.1177/030098588802500205

Mortensen, H., Nielsen, S.S., Berg, P., 2004. Genetic Variation and Heritability of the Antibody Response to Mycobacterium avium subspecies paratuberculosis in Danish Holstein Cows. J. Dairy Sci. 87, 2108–2113. doi:10.3168/jds.S0022-0302(04)70029-6

Mortier, R.A., Barkema, H.W., Bystrom, J.M., Illanes, O., Orsel, K., Wolf, R., Atkins, G., De Buck, J., 2013. Evaluation of age-dependent susceptibility in calves infected with two doses of Mycobacterium avium subspecies paratuberculosis using pathology and tissue culture. Vet. Res. 44, 94. doi:10.1186/1297-9716-44-94

Mortier, R.A.R., Barkema, H.W., De Buck, J., 2015. Susceptibility to and diagnosis of Mycobacterium avium subspecies paratuberculosis infection in dairy calves: A review. Prev. Vet. Med. 121, 189–198. doi:10.1016/j.prevetmed.2015.08.011

Naser, S., Ghobrial, G., Romero, C., Valentine, J.F., 2004. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. Lancet 364, 1039–44. doi:10.1016/S0140-6736(04)17058-X

National Action Johne's Group, 2017. Action Johne's [WWW Document]. URL http://www.actionjohnesuk.org (accessed 2.27.17).

National Action Johne's Group, 2014. Know your Johne's status and how to control it.

National Milk Laboratory, 2017. NML Testing Services [WWW Document]. URL http://www.nationalmilklaboratories.co.uk/johnes/ (accessed 2.25.17).

National Milk Records, 2017. Herdwise [WWW Document]. URL https://www.nmr.co.uk/disease/herdwise (accessed 2.25.17).

Nekouei, O.A., Sanchez, J., Keefe, G.P., 2015. Carryover of bovine leukemia virus antibodies in samples from shared milk meters. J. Dairy Sci. 98, 5274–5279. doi:10.3168/jds.2014-9036

Ng-Kwai-Hang, K.F., Hayes, J.F., Moxley, J.E., Monardes, H.G., 1984. Variability of Test-Day Milk Production and Composition and Relation of Somatic Cell Counts with Yield and Compositional Changes of Bovine Milk. J. Dairy Sci. 67, 361–366. doi:10.3168/jds.S0022-0302(84)81309-0

Nielsen, S., Toft, N., 2009. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev. Vet. Med. 88, 177–197.

Nielsen, S.S., 2011. Dairy farmers' reasons for participation in the Danish control programme on bovine paratuberculosis. Prev. Vet. Med. 98, 279–283. doi:10.1016/j.prevetmed.2010.12.005

Nielsen, S.S., 2010. Immune-based Diagnosis of Paratuberculosis, in: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control.

CAB International, Oxfordshire, pp. 284–293.

Nielsen, S.S., Bjerre, H., Toft, N., 2008. Colostrum and Milk as Risk Factors for Infection with Mycobacterium avium subspecies paratuberculosis in Dairy Cattle. J. Dairy Sci. 91, 4610–4615. doi:10.3168/jds.2008-1272

Nielsen, S.S., Enevoldsen, C., Gr??hn, Y.T., 2002. The Mycobacterium avium subsp. paratuberculosis ELISA response by parity and stage of lactation. Prev. Vet. Med. 54, 1–10.

Nielsen, S.S., Ersbøll, a K., 2006. Age at occurrence of Mycobacterium avium subspecies paratuberculosis in naturally infected dairy cows. J. Dairy Sci. 89, 4557–4566. doi:10.3168/jds.S0022-0302(06)72505-X

Nielsen, S.S., Gröhn, Y.T., Quaas, R.L., Agger, J.F., 2002. Paratuberculosis in Dairy Cattle: Variation of the Antibody Response in Offspring Attributable to the Dam. J. Dairy Sci. 85, 406–412. doi:10.3168/jds.S0022-0302(02)74088-5

Nielsen, S.S., Toft, N., 2008. Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. Vet. Microbiol. 129, 217–35. doi:10.1016/j.vetmic.2007.12.011

Nielsen, S.S., Toft, N., 2006. Age-Specific Characteristics of ELISA and Fecal Culture for Purpose-Specific Testing for Paratuberculosis. J. Dairy Sci. 89, 569–579. doi:10.3168/jds.S0022-0302(06)72120-8

Nielsen, S.S., Toft, N., Okura, H., 2013. Dynamics of specific anti-Mycobacterium avium subsp. paratuberculosis antibody response through age. PLoS One 8, e63009. doi:10.1371/journal.pone.0063009

NML, 2011. Johne's Disease testing toolkit: frequently asked questions.

NMR, n.d. JOHNE ' S DISEASE: A GUIDE TO SURVEILLANCE.

North Dakota Department of Agriculture, 2017. Johne's Disease [WWW Document].

O'Reilly, C.E., O'Connor, L., Anderson, W., Harvey, P., Grant, I.R., Donaghy, J., Rowe, M., O'Mahony, P., 2004. Surveillance of bulk raw and commercially pasteurized cows' milk from approved Irish liquid-milk pasteurization plants to determine the incidence of Mycobacterium paratuberculosis. Appl. Environ. Microbiol. 70, 5138–5144. doi:10.1128/AEM.70.9.5138-5144.2004

Ogus, A.C., Yoldas, B., Ozdemir, T., Uguz, A., Olcen, S., Kaser, I., Coskun, M., Cilli, A., Yegin, O., 2004. The Arg753Gln polymorphism of the human Toll-like receptor 2 gene in tuberculosis disease. Eur. Respir. J. 23, 219–223. doi:10.1183/09031936.03.00061703

Olde Riekerink, R.G.M., Barkema, H.W., Veenstra, W., Berg, F.E., Stryhn, H., Zadoks, R.N., 2007. Somatic cell count during and between milkings. J. Dairy Sci. 90, 3733–41. doi:10.3168/jds.2007-0001

Olivier, K.N., Weber, D.J., Wallace, R.J., Faiz, A.R., Lee, J.H., Zhang, Y., Brown-Elliot, B.A., Handler, A., Wilson, R.W., Schechter, M.S., Edwards, L.J., Chakraborti, S., Knowles, M.R., 2003. Nontuberculous mycobacteria: I: Multicenter prevalence study in cystic fibrosis. Am. J. Respir. Crit. Care Med. 167, 828–834. doi:10.1164/rccm.200207-678OC

Olsen, I., Storset, A., 2001. Innate IFN-γ production in cattle response to MMP14, a secreted protein from Mycobacterium avium subsp. paratuberculosis. Scand. J. Immunol. 54, 306–313.

Ordolff, D., 1997. Experiments on automatic preparation of milk samples in connection with milking robots. Comput. Electron. Agric. 17, 133–137. doi:10.1016/S0168-1699(96)01226-4

Orpin, P., Sibley, D., 2014. Managing endemic infectious disease in cattle herds. Vet. Rec. 174, 193–195. doi:10.1136/vr.g1620

Osterstock, J.B., Fosgate, G.T., Norby, B., Manning, E.J.B., Collins, M.T., Roussel, A.J., 2007. Contribution of environmental mycobacteria to false-positive serum ELISA results for paratuberculosis. J. Am. Vet. Med. Assoc. 230.

Paolicchi, F., Cirone, K., Morsella, C., Gioffre, A., 2012. First Isolation of Mycobacterium Avium Subsp Paratuberculosis from Commercial Pasteurized Milk in Argentina. Brazilian J. Microbiol. 244, 1034–1037. doi:10.1038/321119b0

Patel, D., Danelishvili, L., Yamazaki, Y., Alonso, M., Paustian, M.L., Bannantine, J.P., Meunier-Goddik, L., Bermudez, L.E., 2006. The ability of Mycobacterium avium subsp. paratuberculosis to enter bovine epithelial cells is influenced by preexposure to a hyperosmolar environment and intracellular passage in bovine mammary epithelial cells. Infect. Immun. 74, 2849–2855. doi:10.1128/IAI.74.5.2849-2855.2006

Payne, J., Rankin, J., 1961. The pathogenesis of experimental Johne's disease in calves. Res. Vet. Sci. 2, 167–174.

PBD Biotech Ltd, 2017. Technology [WWW Document]. URL http://www.pbdbio.com (accessed 2.24.17).

Pearce, L.E., Truong, H.T., Crawford, R. a., Yates, G.F., Cavaignac, S., De Lisle, G.W., 2001. Effect of Turbulent-Flow Pasteurization on Survival of Mycobacterium avium subsp. paratuberculosis Added to Raw Milk. Appl. Environ. Microbiol. 67, 3964–3969. doi:10.1128/AEM.67.9.3964-3969.2001

Peck, D.F., Grant, S., McArthur, W., Godden, D., 2002. Psychological impact of

foot-and-mouth disease on farmers. J. Ment. Heal. 11, 523–531. doi:10.1080/09638230020023877

Pin, D., Guérin-faublée, V., Dumitrescu, O., Flandrois, J., Lina, G., 2014. Species Related from Cows with Bovine Nodular Thelitis 20, 2111–2114.

Pouillot, R., Dufour, B., Durand, B., 2004. Original article A deterministic and stochastic simulation model for intra-herd paratuberculosis transmission. Vet. Res. 35, 53–68. doi:10.1051/vetres

Quist, M.A., LeBlanc, S.J., Hand, K.J., Lazenby, D., Miglior, F., Kelton, D.F., 2008. Milking-to-Milking Variability for Milk Yield, Fat and Protein Percentage, and Somatic Cell Count. J. Dairy Sci. 91, 3412–3423. doi:10.3168/jds.2007-0184

Rademaker, J.L.W., Vissers, M.M.M., Te Giffel, M.C., 2007. Effective heat inactivation of Mycobacterium avium subsp. paratuberculosis in raw milk contaminated with naturally infected feces. Appl. Environ. Microbiol. 73, 4185–4190. doi:10.1128/AEM.00326-07

Rajeev, S., Shulaw, W., Berghaus, R., Zhang, Y., Byrum, B., 2006. A testing scheme for the detection of Mycobacterium avium subsp. paratuberculosis in bovine feces utilizing the ESP para-JEM liquid culture system. J Vet Diagn Invest 18, 529–535.

Rhodes, G., Henrys, P., Thomson, B.C., Pickup, R.W., 2013. Mycobacterium avium subspecies paratuberculosis is widely distributed in British soils and waters: implications for animal and human health. Environ. Microbiol. 15, 2761–74. doi:10.1111/1462-2920.12137

Rhodes, G., Richardson, H., Hermon-Taylor, J., Weightman, A., Higham, A., Pickup, R., 2014. Mycobacterium avium Subspecies paratuberculosis: Human Exposure through Environmental and Domestic Aerosols. Pathogens 3, 577–595. doi:10.3390/pathogens3030577

Ridge, S.E., Heuer, C., Cogger, N., Heck, a., Moor, S., Baker, I.M., Vaughan, S., 2010. Herd management practices and the transmission of Johne's disease within infected dairy herds in Victoria, Australia. Prev. Vet. Med. 95, 186–197. doi:10.1016/j.prevetmed.2010.05.001

Ritter, C., Kwong, G.P.S., Wolf, R., Pickel, C., Slomp, M., Flaig, J., Mason, S., Adams, C.L., Kelton, D.F., Jansen, J., De Buck, J., Barkema, H.W., 2015. Factors associated with participation of Alberta dairy farmers in a voluntary, management-based Johne's disease control program. J. Dairy Sci. 98, 7831–7845. doi:http://dx.doi.org/10.3168/jds.2015-9789

Roussel, A.J., Fosgate, G.T., Manning, E.J.B., Collins, M.T., 2007. Association of fecal shedding of mycobacteria with high ELISA-determined seroprevalence for paratuberculosis in beef herds. J. Am. Vet. Med. Assoc. 230, 890–895. doi:10.2460/javma.230.6.890

Rowe, M.T., Grant, I.R., Dundee, L., Ball, H.J., 2000. Heat resistance of Mycobacterium avium subsp. paratuberculosis in milk. Irish J. Agric. Food Res. 39, 203–208.

Ryan, T.J., Livingstone, P.G., Ramsey, D.S.L., De Lisle, G.W., Nugent, G., Collins, D.M., Buddle, B.M., 2006. Advances in understanding disease epidemiology and implications for control and eradication of tuberculosis in livestock: The experience from New Zealand. Vet. Microbiol. 112, 211–219. doi:10.1016/j.vetmic.2005.11.025

SAC consulting, 2016. Farm Animal Price List 2016/2017 [WWW Document]. URL http://www.sruc.ac.uk/downloads/file/1922/farm_animal_services_price_list_201516 (accessed 12.31.16).

Salgado, M., Collins, M.T., Salazar, F., Kruze, J., Bölske, G., Söderlund, R., Juste, R., Sevilla, I.A., Biet, F., Troncoso, F., Alfaro, M., 2011. Fate of Mycobacterium avium subsp. paratuberculosis after application of contaminated dairy cattle manure to agricultural soils. Appl. Environ. Microbiol. 77, 2122–2129. doi:10.1128/AEM.02103-10

Scanu, A.M., Bull, T.J., Cannas, S., Sanderson, J.D., Sechi, L. a., Dettori, G., Zanetti, S., Hermon-Taylor, J., 2007. Mycobacterium avium subspecies paratuberculosis infection in cases of irritable bowel syndrome and comparison with Crohn's disease and Johne's disease: Common neural and immune pathogenicities. J. Clin. Microbiol. 45, 3883–3890. doi:10.1128/JCM.01371-07

Schlesinger, L., Horwitz, M., 1991. Phagocytosis of Mycobacterium leprae by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of this bacte. J. Immunol. 147, 1983–94.

Schwartz, T., Kalmbach, S., Hoffmann, S., Szewzyk, U., Obst, U., 1998. PCR-based detection of mycobacteria in biofilms from a drinking water distribution system. J. Microbiol. Methods 34, 113–123. doi:10.1016/S0167-7012(98)00081-5

Seitz, S., Heider, L., Heuston, W., Bech-Nielsen, S., Rings, D., Spangler, L., 1989. Bovine fetal infection with Mycobacterium paratuberculosis. J. Am Vet Med Assoc 194, 1423–6.

Shaughnessy, L.J., Smith, L. a, Evans, J., Anderson, D., Caldow, G., Marion, G., Low, J.C., Hutchings, M.R., 2013. High prevalence of paratuberculosis in rabbits is associated with difficulties in controlling the disease in cattle. Vet. J. 198, 267–70. doi:10.1016/j.tvjl.2013.08.030

Shimizu, K., Hirose, T., Sato, M., Tsukaamura, M., 1977. Isolation of Acid-Fast Organisms Resembling from a Lesion of Bovine Nodular Mycobacterium

Thelitis vaccae Kiheiji SHimizu , Tsuneo HIROSE , Motoyoshi Department of Microbiology , Obihiro University of Agriculture and Veterinary Medicine , Obihiro , and. Microbiol. Immunol. 21, 469–472.

Sigurethardóttir, O.G., Valheim, M., Press, C.M., 2004. Establishment of Mycobacterium avium subsp. paratuberculosis infection in the intestine of ruminants. Adv. Drug Deliv. Rev. 56, 819–34. doi:10.1016/j.addr.2003.10.032

Singh, S. V., Singh, A. V., Singh, R., Sandhu, K.S., Singh, P.K., Sohal, J.S., Gupta, V.K., Vihan, V.S., 2007. Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal-PCR for the diagnosis of bovine Johne's disease (BJD) in India. Comp. Immunol. Microbiol. Infect. Dis. 30, 175–186. doi:10.1016/j.cimid.2006.12.002

Siqueira, F.M., Lopes, C.E., Snell, G.G., Gomes, M.J.P., 2016. Identification of Mycobacterium smegmatis in Bovine Mastitis. Acta Sci. Vet. 44, 1–4.

Slana, I., Kralik, P., Kralova, A., Pavlik, I., 2008. On-farm spread of Mycobacterium avium subsp. paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int. J. Food Microbiol. 128, 250–257. doi:10.1016/j.ijfoodmicro.2008.08.013

Slana, I., Paolicchi, F., Janstova, B., Navratilova, P., Pavlik, I., 2008. Detection methods for Mycobacterium avium subsp . paratuberculosis in milk and milk products : a review. Vet. Med. 53, 283–306.

Smith, R.L., Grohn, Y.T., Pradhan, A.K., Whitlock, R.H., Van Kessel, J.S., Smith, J.M., Wolfgang, D.R., Schukken, Y.H., 2009. A longitudinal study on the impact of Johne's disease status on milk production in individual cows. J. Dairy Sci. 92, 2653–61. doi:10.3168/jds.2008-1832

Smyth, G., Verbyla, A., 1996. A conditional likelihood approach to residual maximum likelihood estimation in generalized linear models. J. R. Stat. Soc. 58, 565–572.

Speed, T.P., 2004. Restricted Maximum Likelihood (REML), in: Encyclopedia of Statistical Sciences.

Stabel, J.R., 2008. Pasteurization of colostrum reduces the incidence of paratuberculosis in neonatal dairy calves. J. Dairy Sci. 91, 3600–3606. doi:10.3168/jds.2008-1107

Stabel, J.R., 2001. On-Farm Batch Pasteurization Destroys Mycobacterium paratuberculosis in Waste Milk. J. Dairy Sci. 84, 524–527. doi:10.3168/jds.S0022-0302(01)74503-1

Stabel, J.R., Bradner, L., Robbe-Austerman, S., Beitz, D.C., 2014. Clinical disease and stage of lactation influence shedding of Mycobacterium avium subspecies paratuberculosis into milk and colostrum of naturally

infected dairy cows. J. Dairy Sci. 97, 6296–6304. doi:10.3168/jds.2014-8204

Stabel, J.R., Hurd, S., Calvente, L., Rosenbusch, R.F., 2004. Destruction of Mycobacterium paratuberculosis, Salmonella spp., and Mycoplasma spp. in Raw Milk by a Commercial On-Farm High-Temperature, Short-Time Pasteurizer. J. Dairy Sci. 87, 2177–2183. doi:10.3168/jds.S0022-0302(04)70038-7

Stabel, J.R., Wells, S.J., Wagner, B. a, 2002. Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds. J. Dairy Sci. 85, 525–31. doi:10.3168/jds.S0022-0302(02)74104-0

Stanley, E.C., Mole, R.J., Smith, R.J., Glenn, S.M., Barer, M.R., McGowan, M., Rees, C.E.D., 2007. Development of a new, combined rapid method using phage and PCR for detection and identification of viable Mycobacterium paratuberculosis bacteria within 48 hours. Appl. Environ. Microbiol. 73, 1851–7. doi:10.1128/AEM.01722-06

Steele, J.H., 2000. History, trends, and extent of pasteurization. Javma 217, 175–178.

Stevenson, K., Alvarez, J., Bakker, D., Biet, F., de Juan, L., Denham, S., Dimareli, Z., Dohmann, K., Gerlach, G.F., Heron, I., Kopecna, M., May, L., Pavlik, I., Sharp, J.M., Thibault, V.C., Willemsen, P., Zadoks, R.N., Greig, A., 2009. Occurrence of Mycobacterium avium subspecies paratuberculosis across host species and European countries with evidence for transmission between wildlife and domestic ruminants. BMC Microbiol. 9, 212. doi:10.1186/1471-2180-9-212

Stewart, D., Vaughan, J., Stiles, P., Noske, P., Tizard, M., Prowse, S., Michalski, W., Butler, K., Jones, S., 2007. A long-term bacteriological and immunological study in Holstein-Friesian cattle experimentally infected with Mycobacterium avium subsp. paratuberculosis and necropsy culture results for Holstein-Friesian cattle, Merino sheep and Angora goats. Vet. Microbiol. 122, 83–96. doi:10.1016/j.vetmic.2006.12.030

Sweeney, R.W., 2011. Pathogenesis of Paratuberculosis. Vet. Clin. North Am. - Food Anim. Pract. 27, 537–546. doi:10.1016/j.cvfa.2011.07.001

Sweeney, R.W., 1996. Transmission of Paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12, 305–312. doi:10.1016/S0749-0720(15)30408-4

Sweeney, R.W., Collins, M.T., Koets, A.P., Mcguirk, S.M., Roussel, A.J., 2012. Paratuberculosis (Johne's Disease) in Cattle and Other Susceptible Species. J. Vet. Intern. Med. 26, 1239–1250. doi:10.1111/j.1939-1676.2012.01019.x

Sweeney, R.W., Uzonna, J., Whitlock, R.H., Habecker, P.L., Chilton, P., Scott, P.,

2006. Tissue predilection sites and effect of dose on Mycobacterium avium subs. paratuberculosis organism recovery in a short-term bovine experimental oral infection model. Res. Vet. Sci. 80, 253–259. doi:10.1016/j.rvsc.2005.07.007

Sweeney, R.W., Whitlock, R.H., Rosenberger, A.E., 1992. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30, 166–171.

Swift, B.M.C., Denton, E.J., Mahendran, S. a., Huxley, J.N., Rees, C.E.D., 2013. Development of a rapid phage-based method for the detection of viable Mycobacterium avium subsp. paratuberculosis in blood within 48 h. J. Microbiol. Methods 94, 175–179. doi:10.1016/j.mimet.2013.06.015

Swift, B.M.C., Rees, C.E.D., 2013. Detecting mycobacteria in cattle blood. Vet. Rec. J. Br. Vet. Assoc. 173, 522–523. doi:10.1136/vr.f7067

Syrstad, O., Ron, I., 1978. Day-to-day variation in cell counts in milk. Nord Vet Med 30, 192–198.

Thomas, H. V, Lewis, G., Thomas, D.R., Salmon, R.L., Chalmers, R.M., Coleman, T.J., Kench, S.M., Morgan-Capner, P., Meadows, D., Sillis, M., Softley, P., 2003. Mental health of British farmers. Occup. Environ. Med. 60, 181-5-6. doi:10.1136/OEM.60.3.181

Thomson, J.R., Mollison, N., Matthews, K.P., 1988. An investigation of mastitis due to S. agalactiae, S. uberis and M. smegmatis in a dairy herd. Vet. Rec.

Thorel, M.-F., Morad, M., Fontaine, J.-J., Gourreau, J.-M., 1990. Bovine Nodular Thelitis: a Clinicopathological Study of 20 Cases. Vet. Dermatol. 1, 165–170. doi:10.1111/j.1365-3164.1990.tb00095.x

Tiwari, A., VanLeeuwen, J.A., McKenna, S.L.B., Keefe, G.P., Barkema, H.W., 2006. Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can. Vet. J. 47, 874–882. doi:10.1515/semi.1969.1.3.339

Tizard, I.R., 2013. Veterinary Immunology, 9th ed. Elsevier Saunders, Missori.

Vaerewijck, M.J.M., Huys, G., Palomino, J.C., Swings, J., Portaels, F., 2005. Mycobacteria in drinking water distribution systems: ecology and significance for human health. FEMS Microbiol. Rev. 29, 911–34. doi:10.1016/j.femsre.2005.02.001

van Roermund, H.J.W., Bakker, D., Willemsen, P.T.J., de Jong, M.C.M., 2007. Horizontal transmission of Mycobacterium avium subsp. paratuberculosis in cattle in an experimental setting: calves can transmit the infection to other calves. Vet. Microbiol. 122, 270–9. doi:10.1016/j.vetmic.2007.01.016

Vial, F., Miguel, E., Johnston, W.T., Mitchell, A., Donnelly, C.A., 2015. Bovine

Tuberculosis Risk Factors for British Herds Before and After the 2001 Foot-and-Mouth Epidemic: What have we Learned from the TB99 and CCS2005 Studies? Transbound. Emerg. Dis. 62, 505–515. doi:10.1111/tbed.12184

Wang, C., Turnbull, B.W., Nielsen, S.S., Gröhn, Y.T., 2011. Bayesian analysis of longitudinal Johne's disease diagnostic data without a gold standard test. J. Dairy Sci. 94, 2320–8. doi:10.3168/jds.2010-3675

Weber, M.F., Kogut, J., de Bree, J., van Schaik, G., Nielen, M., 2010. Age at which dairy cattle become Mycobacterium avium subsp. paratuberculosis faecal culture positive. Prev. Vet. Med. 97, 29–36. doi:10.1016/j.prevetmed.2010.07.004

Wetzstein, M., Greenfield, J., 1992. Mastitis caused by a Mycobacterium sp. Can. Vet. J. 33, 826.

Whaley, S.E., Sigman, M., Neumann, C., Bwibo, N., Guthrie, D., Weiss, R.E., Alber, S., Murphy, S.P., 2003. Animal Source Foods to Improve Micronutrient Nutrition and Human Function in Developing Countries. J. Nutr 133, 3965–3971. doi:0022-3166/03

Whan, L., Grant, I.R., Rowe, M.T., 2006. Interaction between Mycobacterium avium subsp. paratuberculosis and environmental protozoa. BMC Microbiol. 6, 63. doi:10.1186/1471-2180-6-63

White, F., Rattray, E.A.S., 1965. Diurnal Variation in the Cell of Cows ' Milk Content.

Whitlock, R.H., Buergelt, C., 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North Am. Food Anim. Pract. 12, 345–356. doi:10.1016/S0749-0720(15)30410-2

Whitlock, R.H., Wells, S.J., Sweeney, R.W., Van Tiem, J., 2000. ELISA and fecal culture for paratuberculosis (Johne ' s disease): sensitivity and specificity of each method. Vet. Microbiol. 77, 387–398.

Whitney, H., Priddle, F., 1992. Mastitis caused by a Mycobacterium sp. in a dairy herd. Can. Vet. J. 33, 344. doi:10.1016/j.trjl.2015.06.016.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, I.B., Reddacliff, L. a, 2004. Survival and Dormancy of Mycobacterium avium subsp. paratuberculosis in the Environment. Appl. Environ. Microbiol. 70, 2989–3004. doi:10.1128/AEM.70.5.2989

Whittington, R.J., Windsor, P. a, 2009. In utero infection of cattle with Mycobacterium avium subsp. paratuberculosis: a critical review and meta-analysis. Vet. J. 179, 60–9. doi:10.1016/j.tvjl.2007.08.023

Williams-Bouyer, N., Yorke, R., Lee, H.I., Woods, G.L., 2000. Comparison of the BACTEC MGIT 960 and ESP culture system II for growth and detection of

mycobacteria. J. Clin. Microbiol. 38, 4167–4170.

Windsor, P. a, Whittington, R.J., 2010. Evidence for age susceptibility of cattle to Johne's disease. Vet. J. 184, 37–44. doi:10.1016/j.tvjl.2009.01.007

Wolf, R., Orsel, K., De Buck, J., Barkema, H.W., 2015. Calves shedding Mycobacterium avium subspecies paratuberculosis are common on infected dairy farms. Vet. Res. 46, 71. doi:10.1186/s13567-015-0192-1

Woodbine, K. a., Schukken, Y.H., Green, L.E., Ramirez-Villaescusa, A., Mason, S., Moore, S.J., Bilbao, C., Swann, N., Medley, G.F., 2009. Seroprevalence and epidemiological characteristics of Mycobacterium avium subsp. paratuberculosis on 114 cattle farms in south west England. Prev. Vet. Med. 89, 102–109. doi:10.1016/j.prevetmed.2009.02.005

Woods, G.L., Washington, J. a, 2011. Mycobacteria other than Mycobacterium tuberculosis: review of microbiologic and clinical aspects. Rev. Infect. Dis. 9, 275–294. doi:10.1093/clinids/9.2.275

Wu, C., Schmoller, S.K., Bannantine, J.P., Eckstein, T.M., Inamine, J.M., Livesey, M., Albrecht, R., Talaat, A.M., 2009. A novel cell wall lipopeptide is important for biofilm formation and pathogenicity of Mycobacterium avium subspecies paratuberculosis. Microb. Pathog. 46, 222–230. doi:10.1016/j.micpath.2009.01.010

ANNEX

PRESENTATIONS AND PUBLICATIONS

Oral Presentations

SfAM Annual Conference; Jul 2014	Impact of new methods of detecting *Mycobacterium avium* subspecies *paratuberculosis* on control of Johne's disease in cattle
Scarsdale Vets Johne's disease workshop; Jan 2015	Can bacteriophage help us to control Johne's Disease in Cattle?
SfAM Early Careers Scientists Conference; Oct 2015	Can bacteriophage help us to control Johne's Disease in Cattle?
International Colloquium for Paratuberculosis; Jun 2016	Comparison of a Phage-PCR assay to culture and ELISA for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle

Posters

SfAM Annual Conference; Jul 2014	Impact of new methods of detecting *Mycobacterium avium* subspecies *paratuberculosis* on control of Johne's disease in cattle
SfAM Early Careers Autumn Meeting; Oct 2014	Can bacteriophage help us to control Johne's Disease in Cattle?
SRUC Conference; Mar 2015	Impact of new methods of detecting *Mycobacterium avium* subspecies *paratuberculosis* on control of Johne's disease in cattle
AVTRW Annual Conference; Sept 2015	Can Bacteriophage Help us to Control Johne's Disease in Cattle?

Papers

Swift, BMC., Gerrard, ZE., Huxley, JN., and Rees, CED. (2014) Factors Affecting Phage D29 Infection: A Tool to Investigate Different Growth States of Mycobacteria. PLoS ONE 9(9): e106690. https://doi.org/10.1371/journal.pone.0106690

Gerrard, ZE., Swift, BMC., Botsaris, G., Davidson, R., Hutchings, M., Huxley, JN., and Rees, CED. (submitted) Survival of Mycobacterium avium subspecies paratuberculosis in retail pasteurised milk. Food Microbiology

Paper title: Impact of new methods of detecting *Mycobacterium avium* subspecies *paratuberculosis* on control of Johne's disease in cattle

Zara Gerrard[1,3], Ross Davidson[3], Mike Hutchings[3], Jon Huxley[2] and Catherine Rees[1]
[1] School of Biosciences and [2] School of Veterinary Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD; [3] Animal & Veterinary Sciences, SRUC, Roslin Institute Building, Easter Bush, Midlothian EH25 9RG

Aims

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease in cattle and is associated with Crohn's disease in humans. The organism is slow growing (up to 18 weeks), making rapid detection problematic. The aim of this study is to generate data to model the impact of a rapid bacteriophage-based assay for the detection of MAP on control practises used within the dairy industry and also gain a better estimate of the viable MAP levels in retail pasteurised milk.

Methods and Results

Johne's disease is endemic within the UK, but prevalence estimations are difficult to establish due to the lack of sensitivity in diagnostics. We have developed a bacteriophage-based assay for the detection of MAP in raw milk. In this study the results will be compared to those of standard milk ELISA tests. Results indicate that individuals who are consistently milk-ELISA positive are shedding high-levels of MAP into the milk, however, negative cattle are also shedding.
The phage assay is also being used to survey pasteurised milk obtained from commercial retailers, as milk is the most common food vehicle for MAP. This method allows live-dead differentiation, therefore only detecting viable cells. We can detect less than 10 MAP cells per 25 ml milk so is more sensitive than culture-based methods. Data from the survey of milk samples indicate that the prevalence is likely to be less than 5%.

Conclusions

Data from the survey of milk taken from individual cows indicated that high level shedding can occur before a persistent ELISA-positive status is established.

Significance of study

The study will be used to improve herd control of Johne's disease and provide a better estimate of the human exposure to MAP via pasteurised milk. If the new assay can be used to reduce the number of MAP cells entering the food chain, this will reduce the risk of exposure to consumers.

References

Botsaris, G, Slana, I, Liapi, M, Dodd, CER, Economides, C, Rees, CED, and Pavlik, I (2010). Rapid detection methods for viable *Mycobacterium avium*

subspecies *paratuberculosis* in milk and cheese. International Journal of Food Microbiology. **141** pp587-590
Stanley, E, Mole, RJ, Smith, RJ, Glenn, SM, Barer, MR, McGowan, M, and Rees, CED (2007). Development of a new, combined rapid method using phage and PCR for detection and identification of viable *Mycobacterium paratuberculosis* bacteria with 48 hours. Applied and Environmental Microbiology. **73** (6) pp1851-1857
Swift, BMC, Denton, EJ, Mahendran, SA, Huxley, JN, and Rees, CED (2013) Development of a rapid phage-based method for the detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in blood within 48h. Journal of Microbiological Methods. **94** pp175-179
Davidson, RS, McKendrick, IJ, Wood, JC, Marion, G, Greig, A, Stevenson, K, Sharp, M, and Hutchings, MR (2012). Accounting for uncertainty in model-based prevalence estimation: paratuberculosis control in dairy herds. BMC Veterinary Research. **8**

Can bacteriophage help us to control Johne's disease in cattle?

Zara Gerrard[1,3], Ross Davidson[3], Mike Hutchings[3], Jon Huxley[2] and Cath Rees[1]

[1] School of Biosciences and [2]School of Veterinary Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD; [3]Animal & Veterinary Sciences, SRUC, Roslin Institute Building, Easter Bush, Midlothian EH25 9RG

Aims

Johne's disease is a severe wasting condition of cattle which results in chronic diarrhoea, weight loss, swelling of the lower jaw and decrease in milk production. It is caused by *Mycobacterium avium* subspecies *paratuberculosis (*MAP), an extremely slow growing organism - up to 18 weeks - making rapid detection problematic. This bacterium has also been associated with Crohn's disease in humans, making it a bacterium of zoonotic importance. We aim to generate data to model the impact of a rapid bacteriophage-based assay on control practises used within farms and gain a better estimate of the levels of viable MAP in retail pasteurised milk.

Methods and results

Johne's disease is endemic within the UK, but prevalence estimations are difficult to establish due to the lack of sensitivity in diagnostics. This study will use a new, rapid (48h), bacteriophage-based assay for the detection of MAP in raw milk, and compare this to more traditional methods such as milk ELISA alongside milk and faecal culture. Results indicate that the cows who are 'high shedders' are not necessarily those who have positive ELISA results, with some negative animals shedding high levels of MAP. The assay is also being used in an ongoing survey of retail pasteurised milk for viable MAP as the method allows live-dead differentiation of bacteria.

Conclusions

Data from the herd level survey indicates that the assay can be used successfully to identify high shedding animals, but these are not necessarily the same animals as those found positive by ELISA.

Significance of study

The study will be used to improve herd control of Johne's disease and provide a better estimate of the human exposure to MAP via pasteurised milk. The new assay can be used to reduce the bacterial load within herds, and therefore the amount of MAP entering the food chain.

Comparison of a phage-PCR assay to culture and ELISA for the detection of *Mycobacterium avium* subs. *paratuberculosis* in dairy cattle

Gerrard Z.E.[1 2], Swift B.[3], Davidson R.[2], Hutchings M.[2], Huxley J.[3], and Rees C.[2]

[1] University of Nottingham, School of Biosciences, Sutton Bonington Campus, Leics, LE12 5RD

[2] SRUC, Animal and Veterinary Sciences, Roslin Institute Building, Easter Bush, Midlothian, EH15 9RG

[3] University of Nottingham, School of Veterinary Science and Medicine, Sutton Bonington Campus, Leics, LE12 5RD

Due to the limitations caused by the slow growth of the organism, detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and diagnosis of Jonhe's disease is notoriously difficult, with limited sensitivity and specificity being reported. The aim of this cross-sectional study was to determine if enumeration of MAP in milk samples from individual animals using the Phage-PCR method could be used to more readily identify 'high shedders' within a herd. To better understand the results gained, results were compared with standard milk ELISA and milk/faecal culture.

For this study six dairy herds were recruited which varied in size, breed and management practices. Milk samples were collected within a week of the quarterly milk ELISA testing from a random cohort of 100 cows per herd. One sample was sent for MAP culture in an accredited laboratory using ESP Trek, and the other was tested using the Phage-PCR method (Botsaris et al., 2013). Analysis of the results indicates that there is little agreement between the Phage-PCR method and the milk ELISA results, with early results suggesting that the highly positive ELISA cows shed very low levels of mycobacteria into the milk.

A further study was conducted where paired faeces and milk samples were collected from a random 25 cows from each herd. Culture results are still pending, and will be available within 2-3 months. However a small number of milk culture experiments have been completed, and the results indicate that only samples with a high number of mycobacteria detected using the phage-PCR method gave a positive culture result, with samples where less than 100 MAP cells were detected did not give a positive PCR result. This is consistent with the well-characterised problem of decreased sensitivity caused by chemical decontamination of culture samples.

As the culture results become available, we should be able to fully evaluate the value of the phage-PCR method to screen individual animals within a herd to identify high shedders, potentially before they become consistently milk ELISA-positive.

Keywords: phage-PCR, rapid detection, shedding rates, culture, ELISA, dairy cattle

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

SRUC

Impact of new methods of detecting *Mycobacterium avium* subspecies *paratuberculosis* on control of Johne's disease in cattle

Zara Gerrard[1,3], Ross Davidson[3], Mike Hutchings[3], Jon Huxley[2] and Cath Rees[1]

[1] School of Biosciences and [2] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD; [3] Animal & Veterinary Sciences, SRUC, Roslin Institute Building, Easter Bush, Midlothian EH25 9RG

Introduction: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease, a severe wasting disease of cattle and other ruminants, characterised by chronic enteritis, weight loss and drop in milk yield (Fig 1). It is endemic throughout the UK, but a realistic estimation of prevalence is difficult to establish due to the nature of the disease and the lack of sensitivity in current diagnostic tests. An association has been made between MAP and Crohn's disease in humans, with milk seen as the most common food vehicle for this organism.

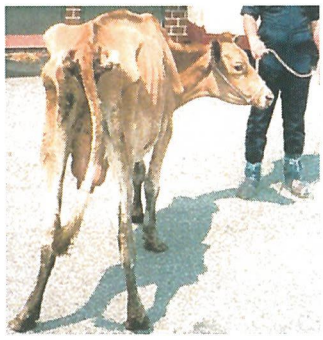
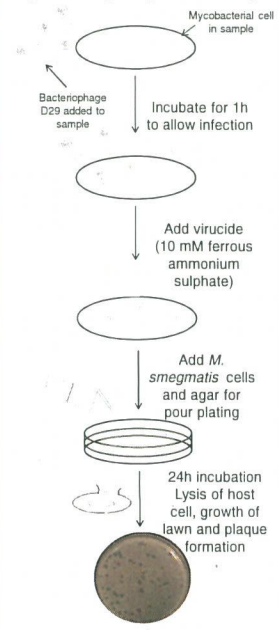


Figure 1. Cow with clinical Johne's disease

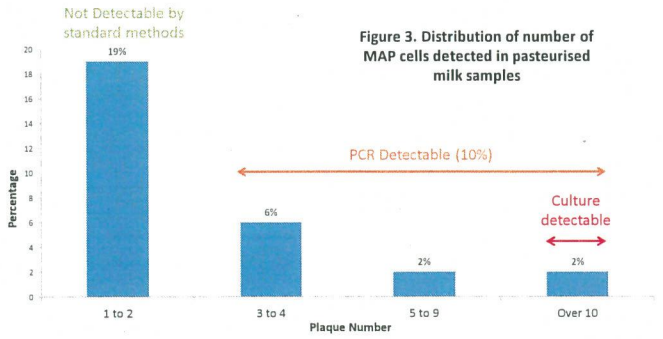
Methods: 50ml of milk was collected and centrifuged, the milk and cream layer removed and resuspended in 7H9 Broth (+ OADC and 2mM $CaCl_2$). The sample was then centrifuged again as a wash step to remove phage inhibitors. To detect the presence of viable Mycobacteria, the phage amplification assay was carried out as shown in Figure 2. DNA was extracted from plaques and IS*900*- specific PCR was then performed to confirm that the mycobacteria detected was MAP (Swift *et al.*, 2013).

Figure 2. Phage Amplification Assay



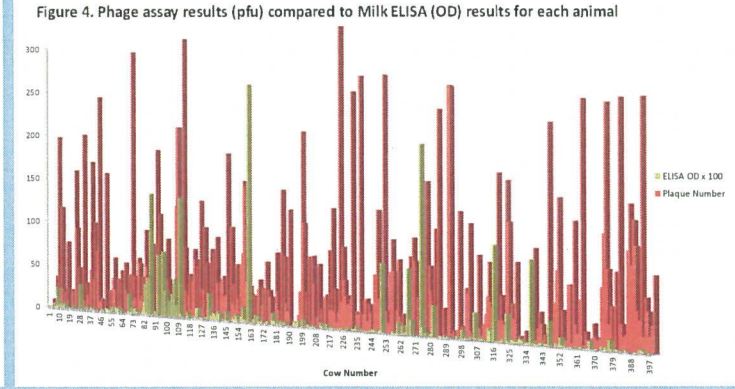
Pasteurised Milk Trial: Pasteurised milk from 14 retail and 5 doorstep sources across Nottinghamshire, Leicestershire and Derbyshire were tested using the phage-PCR assay. The survey results showed that 29% of the samples were MAP-positive. However more than 10 cells were detected in only 2% of the samples, and only these would have been detectable by culture after decontamination. A further 8% contained levels of cells detectable by PCR, which agrees well with the results of Grant *et al.* (2002).

Figure 3. Distribution of number of MAP cells detected in pasteurised milk samples



Individual Cattle Trial: Milk from a herd with high prevalence was tested for MAP using the phage-PCR assay. The results were compared with MAP milk ELISA results (positive cut off = 30; Fig. 4). There appears to be no correlation between the two test results. This supports the hypothesis that some ELISA-positive cows could be fighting off infection as they are showing a high immune response but bacterial shedding is limited.

Figure 4. Phage assay results (pfu) compared to Milk ELISA (OD) results for each animal



Conclusions: The phage assay has proved to be a useful rapid method for the detection of MAP in milk. Preliminary testing within this study shows that this can be used on a large scale for full herd screening to establish herd-level prevalence and incidence rates. Monitoring shedding over time will give a better indication of the health status of the animal, and will help famers control transmission within herds. The test can also be used to establish whether pasteurisation measures are adequate enough to inactive the levels of MAP currently seen in raw milk, and this could now be used for routine milk analysis.

References:

Botsaris *et al* (2010) *Int. J. Food Micro* **141**.
Botsaris *et al* (2013) *Int. J. Food Micro.* **164**.
Davidson *et al* (2012) *BMC Veterinary Research.* **8**
Grant *et al.* (2002) *Appl. Enviro. Bio.* **68**
Stanley *et al* (2007) *Appl. Environ. Microbiol.* **73**.
Swift *et al* (2013) *J. Microbiol. Meths.* **94**.

Zara Gerrard
PhD student, UoN and SRUC
stxzeg@nottingham.ac.uk

Can bacteriophage help us to control Johne's Disease in Cattle?

Zara Gerrard[1,3], Ross Davidson[3], Mike Hutchings[3], Jon Huxley[2] and Cath Rees[1]

[1] School of Biosciences and [2] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD; [3] Animal & Veterinary Sciences, SRUC, Roslin Institute Building, Easter Bush Campus, Midlothian, EH25 9RG

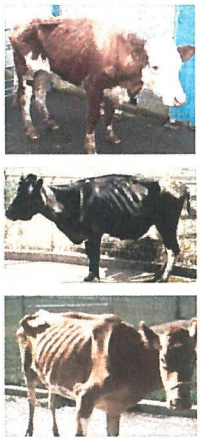


Figure 1. Cattle infected with Johne's disease showing signs of wasting

Introduction; Johne's disease is a severe wasting disease characterised by chronic enteritis, reduction in milk yield and oedema of the lower jaw (Fig. 1). It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) - an extremely slow growing bacterium (up to 18 weeks to form colonies) which is also associated with Crohn's disease in humans. It is endemic within the UK cattle population, but accurate prevalence estimates are limited due to the poor sensitivity in current diagnostic methods. We have developed a rapid (24 h) bacteriophage-based assay for the detection of MAP in milk, which is more sensitive than culture and provides rapid detection of viable MAP cells, unlike ELISA assays which just report on the immune response to MAP. In this study the use of this assay to identify high shedding animals MAP was investigated using milk samples from a herd with a high incidence of MAP milk ELISA-positive animals (10 %).

Methodology; 25 ml of milk from each cow was chilled at 5 °C overnight. This was centrifuged, the cream and milk layers removed and the pellet resuspended in 7H9 broth + OADC and NOA antimicrobial supplement. Viable mycobacteria were detected using the phage amplification assay (Fig. 3). To confirm the detection of MAP, DNA was extracted from plaques and IS*900*-specific PCR performed (Swift *et al.*, 2013). A small number (n=6) samples were also sent for conventional culture along with control samples to which different numbers of MAP were added.

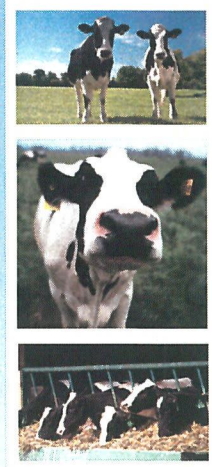


Figure 2. Healthy dairy cattle

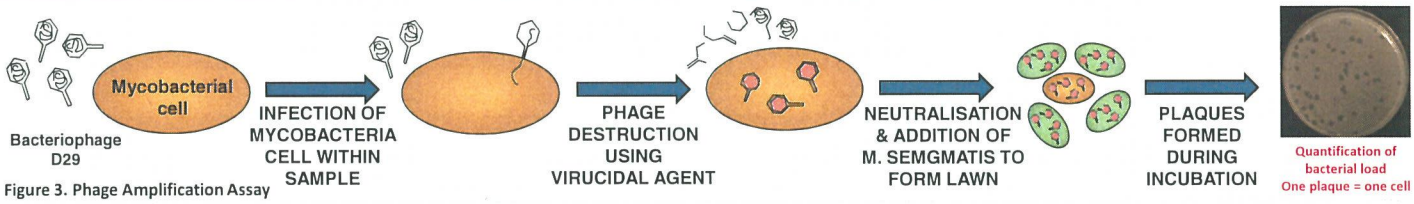


Figure 3. Phage Amplification Assay

Herd Level Results; The levels of MAP in milk from individual animals from a herd was determined using the phage-PCR assay. These results were then compared to the ELISA results (positive cut-off value = 0.3; Fig. 4). There appears to be no correlation between the phage assay result which reports on levels of shedding of viable bacteria into the milk and the MAP milk ELISA test results.

Interestingly a number of cows were identified where no mycobacteria were detected in the milk but gave a positive milk ELISA test result, perhaps indicating animals that are fighting off infection and are showing a high immune response but are controlling bacterial growth. Further sampling as part of a longitudinal study is underway to determine whether the levels of shedding remain stable or fluctuate in the same way that the milk ELISA results are known to fluctuate (three consecutive positive milk ELISA test results are required to identify an infected animal.

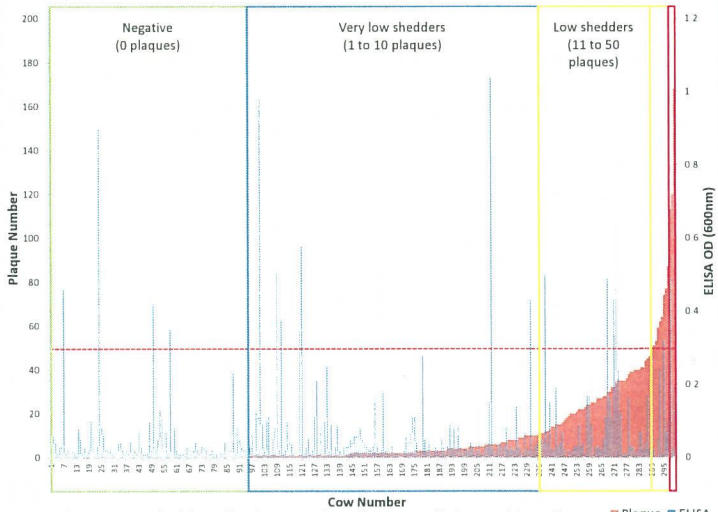


Fig 4. Phage assay results (pfu; red bars) compared to milk ELISA results (OD_{600nm}) for each cow

Table 1: Test comparisons

Cow number/ Sample type	Phage-PCR assay (pfu/25 ml)	Milk ELISA test results (OD_{600nm})	Automated Liquid Culture plus PCR confirmation
0892 Low	32	0.16	-ve
1604 Low	32	0.11	-ve
1599 Low	35	0.64	-ve
1445 High	87	0.07	-ve
1101 High	120	0.49	-ve
2113 High	168	0.06	+ve * (PCR only)
Spiked sample A	10^1 added to milk	n/a	+ve * (PCR only)
Spiked sample B	10^2 added to milk	n/a	+ve (30 d to detection)
Spiked sample C	10^3 added to milk	n/a	+ve (15 d to detection)

* Sample didn't signal positive during automated culture but was PCR positive at sampling after 42 d of culture.
RED = MAP-positive test results,

Comparison of Different Test Results; Table 1 shows a comparison of the results gained for a small number of animals from the herd using the two most popular MAP diagnostic tests (culture and milk ELISA) and the phage-PCR assay. Control samples of milk spiked with a known number of MAP cells were all positive on culture, with the time to detection being shortest for samples with the highest number of cells. Six samples of milk were chosen from either Low (11-50 plaques) or High (>50 plaques) shedding animals. Only two of these gave positive milk ELISA results and only the sample with the highest number of plaques was culture-positive.

Conclusions: The phage-PCR assay appears to a more reliable method to identify cattle that are shedding bacteria into the milk than the conventional methods tested. This preliminary study shows that this method can be used as a practical test to allow full herd screening to determine both herd-level prevalence and to identify shedding animals and this information can be used by famers control transmission within herds. Future sampling as part of a longitudinal study will monitor patterns of shedding over time to try and provide a better indication of the long term health status of the animal.

Zara Gerrard, Joint UoN and SRUC PhD student
stzeg@nottingham.ac.uk

Impact of new methods of detecting *Mycobacterium avium* subspecies *paratuberculosis* on control of Johne's disease in cattle

Zara Gerrard[1,2], Ross Davidson[1], Mike Hutchings[1], Jon Huxley[2] and Cath Rees[2]

1. Scotland's Rural College
West Mains Road
Edinburgh, EH9 3JG, Scotland
Email: Zara.Gerrard@sruc.ac.uk

2. University of Nottingham,
Sutton Bonington Campus,
Leics, LE12 5RD, England

www.sruc.ac.uk

Introduction

Johne's disease is a severe wasting disease characterised by chronic enteritis, reduction in milk yield and oedema of the lower jaw. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) - an extremely slow growing bacterium (up to 18 weeks to form colonies) which is also associated with Crohn's disease in humans. It is endemic within the UK cattle population, but accurate prevalence estimates are limited due to the poor sensitivity in current diagnostic methods. We have developed a rapid (24 h) bacteriophage-based assay for the detection of MAP in milk, which is more sensitive than culture and provides rapid detection of viable MAP cells, unlike ELISA assays which just report on the immune response to MAP.

Methods

A milk sample from each cow was chilled at 5 °C overnight. This was centrifuged, the cream and milk layers removed and the pellet resuspended in 7H9 broth + OADC and NOA antimicrobial supplement. Viable mycobacteria were detected using the phage amplification assay (Fig 1). To confirm the detection of MAP, DNA was extracted from plaques and IS*900*-specific PCR performed (Swift *et al.*, 2013).

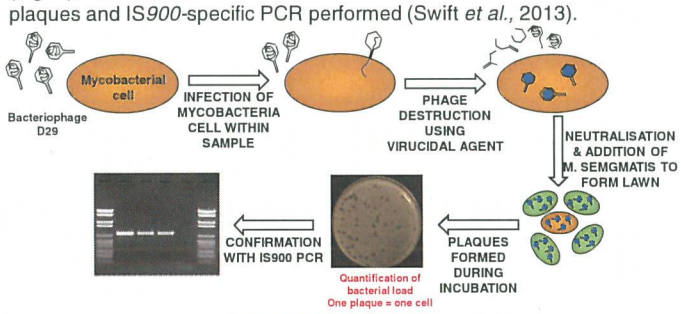


Figure 1. Bacteriophage Assay

Results

A 300 head dairy herd on quarterly milk ELISA testing was visited four times between April 2014 and January 2015. Matched samples were taken for the bacteriophage-based assay as those going for ELISA. The results (Fig 2) show a lack of correlation between ELISA and apparent shedding rates from the phage-based assay. However, there also seemed to be a difference in the range of results over each visit, coupled with trouble gaining PCR positive results some more investigation was required.

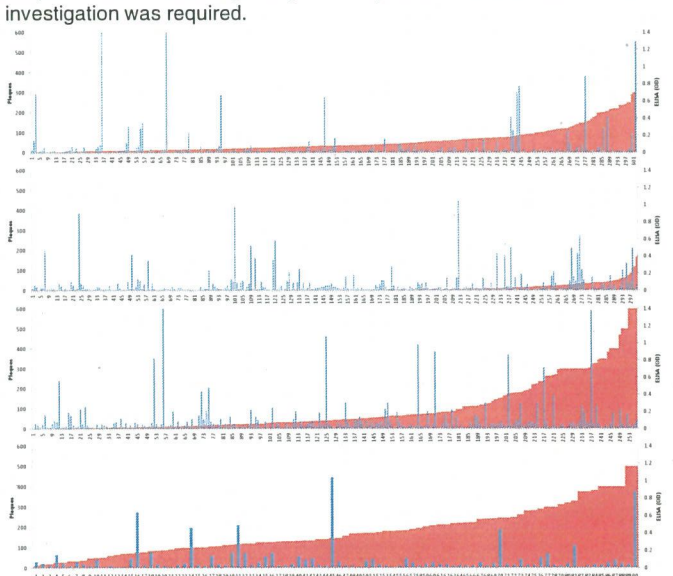


Figure 2. Shedding rates of a herd over 9 months in 2014-15, from top to bottom April, July, October and January. NB. only 100 cows were tested in January.

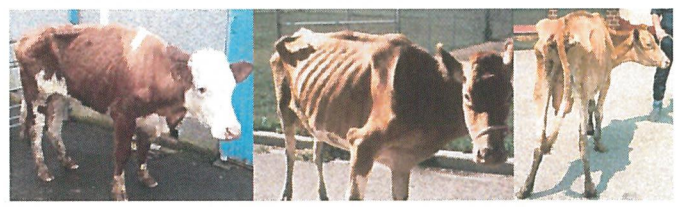


Figure 3. Clinical Johne's Cattle

Table 1. Environmental sampling of milking robot

Location on robot	Phage Assay (pfu/ml)	PCR confirmation
1	7	-ve
2	60	-ve
3	3	-ve
4	2	-ve
5	0	na
6	5	-ve
7	15	+ve
8	38	-ve
9	6	-ve
10	0	na

Environmental sampling was conducted to find out if there was an environmental load of non-specific Mycobacteria in the pipes of the milking machines that could be 'hiding' MAP cells making them harder to find by plaque-PCR (Table 1). All samples apart from one were negative for MAP leading to the conclusion that there was an environmental load of Mycobacteria present on farm.

Table 2 shows a comparison of the results gained for a small number of animals from the herd using the two most popular MAP diagnostic tests (culture and milk ELISA) and the phage-PCR assay. Control samples of milk spiked with a known number of MAP cells were all positive on culture, with the time to detection being shortest for samples with the highest number of cells. Six samples of milk were chosen from either Low (11-50 plaques) or High (>50 plaques) shedding animals. Only two of these gave positive milk ELISA results and only the sample with the highest number of plaques was culture-positive.

Table 2. Test comparisons

Cow number/ Sample type	Phage-PCR assay (pfu/25 ml)	Milk ELISA test results (OD_{600nm})	Automated Liquid Culture plus PCR confirmation
0892 Low	32	0.16	+ve
1604 Low	32	0.11	+ve
1599 Low	35	0.84	+ve
1445 High	87	0.07	+ve
1101 High	120	0.49	+ve
2113 High	183	0.06	+ve * (PCR only)
Spiked sample A	10^1 added to milk	n/a	+ve * (PCR only)
Spiked sample B	10^2 added to milk	n/a	+ve (39 d to detection)
Spiked sample C	10^3 added to milk	n/a	+ve (11 d to detection)

Conclusions

The phage-based assay is a good tool for monitoring MAP shedding rates in dairy cattle, and is more sensitive than the current gold standard in Johne's detection. However, it does have some drawbacks in the fact that it also picks up on environmental contamination of the machines, making MAP confirmation difficult.

To combat this, the assay will be redesigned to remove the plaque picking element, reducing the risk of a false-negative result, and therefore increasing the specificity of the assay.

References

Botsaris, G, Slana, I, Liapi, M, Dodd, CER, Economides, C, Rees, CED, and Pavlik, I (2010). Rapid detection methods for viable *Mycobacterium avium* subspecies *paratuberculosis* in milk and cheese. International Journal of Food Microbiology. **141** pp587-590

Botsaris, G., Liapi, M., Kakogiannis, C., Dodd, CER., and Rees, CED. (2013) 'Detection of *Mycobacterium avium* subspecies *paratuberculosis* in bulk-tank milk by combined phage-PCR assay: Evidence that plaque number is a good predictor of MAP' *International Journal of Food Microbiology* **164**pp76-80

DAVIDSON, R., MCKENDRICK, IJ., WOOD, JC., MARION, G., GREIG, A., STEVENSON, K., SHARP, M., AND HUTCHINGS, MR. 2012. Accounting for uncertainty in model-based prevalence estimation: paratuberculosis control in dairy herds. *BMC Veterinary Research*, 8.Stanley *et al* (2007) *Appl. Environ. Microbiol.* **73**.

Swift, BMC, Denton, EJ, Mahendran, SA, Huxley, JN, and Rees, CED (2013) Development of a rapid phage-based method for the detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in blood within 48h. Journal of Microbiological Methods. **94** pp175-179

Acknowledgements

I would like to acknowledge the work of the farmers in accommodating my study into their daily routines, and for helping with the collection of samples and the team at the University of Nottingham where I am primarily based.

Zara Gerrard
UoN and SRUC PhD Student
stzzeg@nottingham.ac.uk
@ZaraBewizibet

Can Bacteriophage Help us to Control Johne's Disease in Cattle?

Zara Gerrard[1,3], Ross Davidson[3], Mike Hutchings[3], Jon Huxley[2] and Cath Rees[1,1]

School of Biosciences and [2]School of Veterinary Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD; [3]Animal & Veterinary Sciences, SRUC, Roslin Institute Building, Easter Bush, Midlothian EH25 9RG

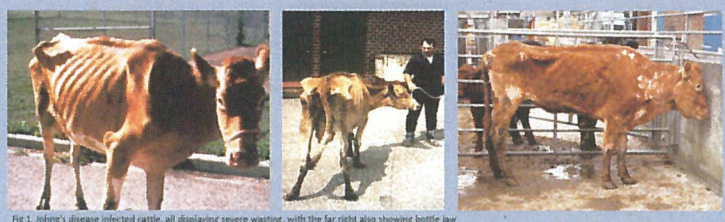


Fig 1. Johne's disease infected cattle, all displaying severe wasting, with the far right also showing bottle jaw

The Disease; Johne's disease is a **severe wasting disease** caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is characterised by **chronic enteritis**, severe weight loss, **reduction in milk yield** and oedema of the lower jaw (bottle jaw).

Diagnostics; Traditional culture methods are normally the gold standard to detect bacteria. MAP is an **extremely slow growing** bacterium (up to 18 weeks to form colonies) which makes detection problematic. So ELISA's are most commonly used to detect infection, but theses detect the immune response rather than the bacteria.

Current Situation; Johne's is **endemic within the UK**[1]; the reduction in milk yield makes it a disease of **high economic importance**. MAP could also be **zoonotic** as it has been associated with Crohn's disease in humans[2].

The Solution; A rapid - **24 h** - bacteriophage-based assay has been developed which **detects viable MAP cells**, rather than an associated immune response.

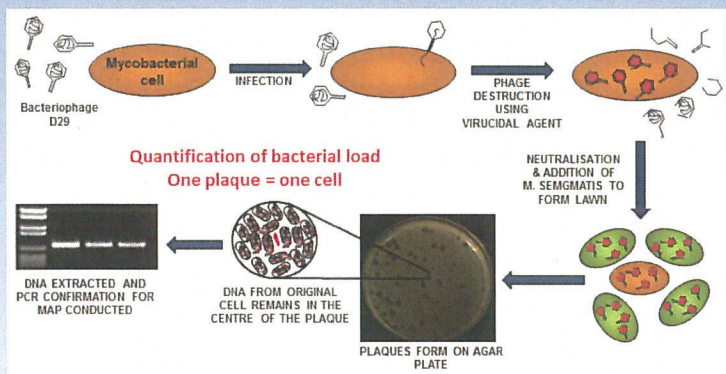


Fig 2. Bacteriophage-based assay carried out on milk samples after a centrifugation wash step

Methodology; Milk was centrifuged, the cream and milk layers removed and the pellet resuspended. Viable mycobacteria from the sample were then detected using the phage amplification assay shown above[3-6]. Results were then compared to culture and ELISA results (below).

Cattle Results; A small cohort of samples and some controls (milk spiked with lab grown culture) were sent for culture alongside the phage assay as shown below. The time to detection was shortest for samples with the highest number of cells. Only **two clinical samples gave positive ELISA results and only the sample with the highest number of plaques was culture-positive.**

Cow number	Phage-PCR assay (pfu/25 ml)	Milk ELISA test results (OD_{600nm})	Automated Liquid Culture plus PCR confirmation
0892	32	0.16	-ve
1604	32	0.11	-ve
1599	35	0.64	-ve
1445	87	0.07	-ve
1101	170	0.49	-ve
2113	168	0.06	+ve * (PCR only)
Spiked sample A	10^1 added to milk	n/a	+ve * (PCR only)
Spiked sample B	10^2 added to milk	n/a	+ve (30 d to detection)
Spiked sample C	10^3 added to milk	n/a	+ve (15 d to detection)

* Sample didn't signal positive during automated culture but was PCR positive at sampling after 42 d of culture.
RED = MAP-positive test results; GREEN = MAP-negative test results

Phage results from 100 cows were also compared to their paired ELISA results (below). The two ELISA thresholds of 'uncertain' and 'positive' are represented by the orange and red lines respectively. This shows that those cows classed as **'positive' by ELISA** are **not necessarily shedding MAP in their milk**.

This has led to many new questions about MAP shedding rates and its **relationship with other diagnostics** (eg culture and blood ELISA) and whether there are **daily changes**, or **changes during milking**.

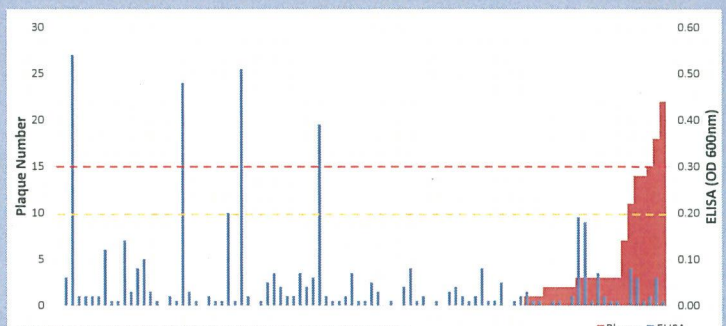
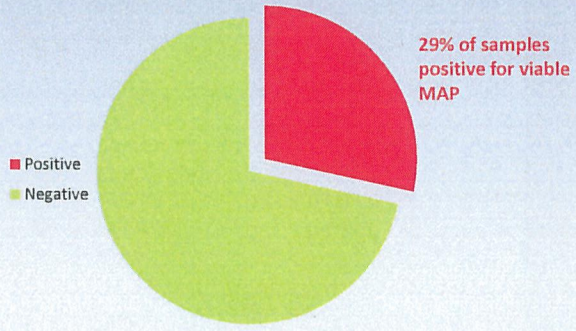


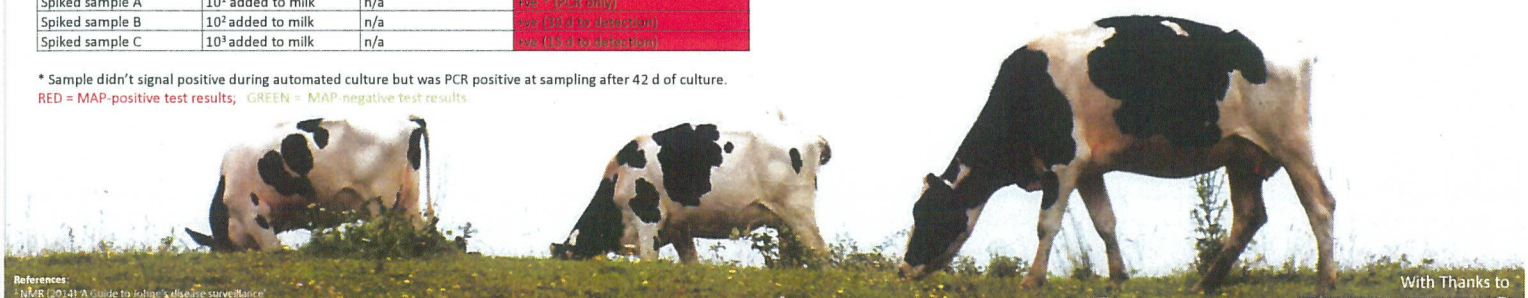
Fig 3. Phage assay results compared to ELISA results from milk samples taken on the same day

Zoonotic Risk; Another question that was asked was how much of this was coming through into the human food chain. So, **100 pasteurised milk samples** from 14 retail and 5 doorstep sources across Nottinghamshire, Leicestershire and Derbyshire were tested using the phage assay (below). The survey showed that **29% were MAP-positive**, which is an increase from the 2% quoted in the FSA survey of 2002[7], however the large increase is most likely due to the **improvement in the sensitivity** of the test, rather than an increase in the amount of Mycobacteria.



The Future; The bacteriophage-based assay has shown to be able to detect MAP in milk from a herd with a known clinical infection. To move the project forward we will be looking at a range of farms using a cross-sectional study to determine if it can be detected in herds that are thought to be negative, so proving that this diagnostic is more sensitive for the detection of MAP. Comparative testing between the current diagnostics and the bacteriophage-based assay will also be continued allowing more insight into the disease and its progression.

Acknowledgements; My supervisors and the support staff from both universities, DEFRA for sponsoring me to attend the conference and finally the farmers and cows for making the sampling possible.



References:
[1] NMR (2014) 'A Guide to Johne's disease surveillance'
[2] Scanu et al (2002) *J. clin. Microbiology* **45**
[3] Stanley et al (2007) *Appl. Environ. Microbiol.* **73**
[4] Botsaris et al (2010) *Int. J. Food Micro* **141**
[5] Botsaris et al (2013) *Int. J. Food Micro.* **164**
[6] Swift et al (2013) *J. Microbiol. Methods* **94**
[7] Grant et al (2002) *Appl. Environ. Mic.* **68**

With Thanks to
The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA
SRUC
defra

Detection of viable *Mycobacterium paratuberculosis* in UK retail pasteurised milk using phage-PCR

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

SRUC

Adam Saeedi[1], Zara Gerrard[1,3], Ross Davidson[3], Mike Hutchings[3], Jon Huxley[2], Ben Swift[2] , Cath Rees[1]

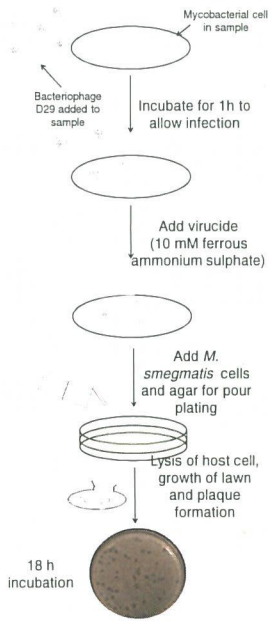
[1] School of Biosciences and [2] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Leicestershire, UK. LE12 5RD;
[3] Animal & Veterinary Sciences, SRUC, Roslin Institute Building, Easter Bush, Midlothian EH25 9RG

Introduction:

Mycobacterium paratuberculosis (MAP) causes Johne's disease, a chronic enteritis of ruminants and has been implicated in the development of Crohn's disease. Milk is a major vertical transmission route within herds and approximately 40-60 % of herds in developed countries are infected. Commercial pasteurisation processes do not completely inactivate MAP therefore pasteurised milk has been highlighted as a key vector for entry of MAP into the human food chain. However the slow growth of this organism prevents the use of standard culture, therefore the use of alternative rapid methods are required to detect its presence.

Figure 1. Cow with Johne's disease showing signs of wasting

Figure 2. Phage Amplification Assay



Background:

We have developed a rapid method that can be used to detect and identify viable MAP in milk (Botsaris *et al.*, 2013). The method uses a bacteriophage-based detection (Fig. 2) followed by PCR identification of the cell detected. The purpose of this study was to carry out a survey of commercial pasteurised semi-skimmed milk using this rapid phage-PCR method to determine the level of viable MAP present in retail pasteurised milk.

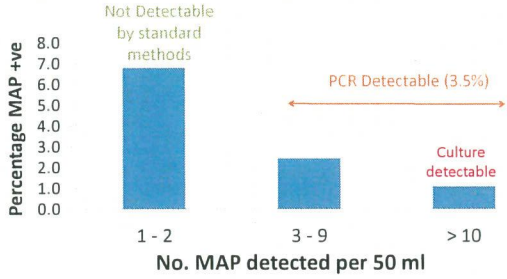
Design of Pasteurised Milk Trial:

Semi-skimmed milk (1.7 % fat) samples (368) were collected between May 2014 and June 2015. The milk was purchased from local retail suppliers across three UK counties (Nottinghamshire, Derbyshire, and Leicestershire). A 50 ml sample was centrifuged and the cream layer and milk removed. The somatic cell pellet was resuspended in 7H9 Broth (supplemented with OADC and 10 mM $CaCl_2$) and washed to remove phage inhibitors. The phage amplification assay was carried out as shown in Fig. 2. DNA was extracted from plaques and IS900-specific PCR used to confirm that MAP was detected (Swift *et al.*, 2013).

Results:

Overall the phage-PCR assay detected viable MAP in 10.3 % (38/368) of samples, defined as the formation of plaques which gave a positive IS900-PCR result.

Figure 3. Distribution of number of MAP cells detected in pasteurised milk samples

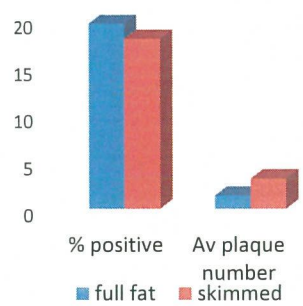


Only 1.1 % of the samples contained more than 10 detectable MAP cells (Fig. 3). The LOD for culture is approx. 30 cells per 40 ml milk) therefore only these are likely to give a positive culture result. Similarly 3.5 % contained more than 2 cells (LOD PCR is approx. 2 cells). These numbers are in line with levels reported in published surveys. The largest number of samples contained 1-2 detectable MAP cells (6.8 %). This number is below the reported limit of detection of either culture or PCR-based detection.

Surprisingly the MAP-positive samples included a number of commercially filtered milk products so this was investigated further.

To determine if MAP was being introduced into the filtered milk with the cream fraction, the number of detectable Mycobacteria in samples of full fat and skimmed milk was compared (Fig. 4). No difference was detected in either the frequency of Mycobacteria detection or number of mycobacteria detected.

Figure 4. Survey of Filtered milk



Significance:

Viable MAP detected were detected at levels consistent with previous surveys of retail milk by culture or PCR. Detection of very low levels (1-2 MAP cells) suggest the rapid phage PCR-method is more sensitive than existing methods. Mycobacteria can also be detected in filtered milk, but does not seem to be introduced with the cream fraction.

References:
Botsaris *et al* (2013) *International Journal of Food Microbiology*. **164**.
Swift *et al* (2013) *J. Microbiol. Meths*. **94**.

OPEN ACCESS Freely available online

PLOS ONE

Factors Affecting Phage D29 Infection: A Tool to Investigate Different Growth States of Mycobacteria

Benjamin M. C. Swift[1]*, Zara E. Gerrard[1], Jonathan N. Huxley[2], Catherine E. D. Rees[1]

1 School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, United Kingdom, **2** School of Veterinary and Medicine Science, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, United Kingdom

Abstract

Bacteriophages D29 and TM4 are able to infect a wide range of mycobacteria, including pathogenic and non-pathogenic species. Successful phage infection of both fast- and slow-growing mycobacteria can be rapidly detected using the phage amplification assay. Using this method, the effect of oxygen limitation during culture of mycobacteria on the success of phage infection was studied. Both D29 and TM4 were able to infect cultures of *M. smegmatis* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) grown in liquid with aeration. However when cultures were grown under oxygen limiting conditions, only TM4 could productively infect the cells. Cell attachment assays showed that D29 could bind to the cells surface but did not complete the lytic cycle. The ability of D29 to productively infect the cells was rapidly recovered (within 1 day) when the cultures were returned to an aerobic environment and this recovery required *de novo* RNA synthesis. These results indicated that under oxygen limiting conditions the cells are entering a growth state which inhibits phage D29 replication, and this change in host cell biology which can be detected by using both phage D29 and TM4 in the phage amplification assay.

Citation: Swift BMC, Gerrard ZE, Huxley JN, Rees CED (2014) Factors Affecting Phage D29 Infection: A Tool to Investigate Different Growth States of Mycobacteria. PLoS ONE 9(9): e106690. doi:10.1371/journal.pone.0106690

Editor: Raymond Schuch, Rockefeller University, United States of America

Received June 20, 2014; **Accepted** July 31, 2014; **Published** September 3, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

* Email: benjamin.swift@nottingham.ac.uk

Introduction

MAP is an extremely slow-growing member of the *Mycobacterium* genus that can take up to 16 weeks to reach detectable levels using traditional culture methods [1]. It is known that pathogenic mycobacteria can persist and survive within macrophages when infecting their host and also that low levels of oxygen can induce dormancy in *M. smegmatis* [2] and *M. tuberculosis* [3]. It is speculated that MAP also has the ability to enter this dormant phase when grown under oxygen limiting conditions [4]. Physiological changes, such as cell wall thickening, have been reported in mycobacteria cells during dormancy [5] and in MAP changes in the expression of 55 proteins involved in hypoxia and starvation were identified [6] all of which may have a role in altering the ability of bacteriophage to infect their host mycobacterial cell.

Some mycobacteriophage, such as D29, TM4, L5 and Bxz2, have been isolated and are found to have a very broad host range so therefore must bind to receptors found on many different mycobacterial cell types [7]. While mycobacteriophage host infection is expected to be strongly determined by the availability of specific cellular receptors, few of these have been identified or studied [8], therefore little is known about the growth conditions needed to ensure that these receptors are expressed to promote good phage infection. In the past bacteriophage have proved to be important tools for interrogating the genetics and physiology of their pathogenic hosts [9]. In mycobacteria, they have been used to rapidly report on the antibiotic resistance of the host cell and rapid phage-based detection methods have been developed [10]. For instance phage amplification assays monitor the rapid growth of the phage which is faster than the growth rate of their mycobacterial host cell. The phage most commonly used to detect MAP is D29. For MAP, this assay has been used as rapid alternative to culture to detect and enumerate MAP in samples of blood, milk and cheese [10–13].

The aim of this investigation was to use the phage amplification assay as a tool to investigate how different growth and storage conditions of mycobacteria affect the host cell physiology, and thereby affect the efficiency of mycobacteriophage infection of the host cells.

Materials and Methods

Bacterial strains, bacteriophage and growth media

Two well described MAP cattle reference strains used were K10 and ATCC 19698 and one clinical cattle isolated kindly gifted by Dr Karen Stevenson; DVL 943.The *Mycobacterium smegmatis* strain used was mc^2155, which is used routinely in phage assays [14]. All liquid cultures were grown in Media Plus (MP; Middlebrook 7H9/OADC [Becton Dickenson] supplemented with 2 mM $CaCl_2$ [15]) or grown on Middlebrook 7H10/OADC agar slopes. For growth of MAP the media was supplemented with Mycobactin J (2 $\mu g\ \mu l^{-1}$; Synbiotics Corporation, France).

Bacteriophage D29 and TM4 were propagated with *M. smegmatis* on 7H10 agar [15].

Detection, enumeration and antibiotic sensitivity testing of MAP

Detection, enumeration and antibiotic sensitivity testing was carried out according to [10]. Briefly, to perform the phage amplification assay, 1 ml samples containing mycobacteria were mixed with 1×10^8 mycobacteriophage D29 (100 µl) and incubated for 1 h. After this time any remaining extracellular phage were inactivated using a virucide treatment (100 µl 10 mM ferrous ammonium sulphate) for 5 min. The virucide was then neutralised by dilution using 5 ml MP and the phage-infected cells were plated in a lawn of fast growing *M. smegmatis* (1 ml, 10^7 CFU ml^{-1}) using soft agar (0.8% w/v). Lysis of the infected cells at the end of the lytic cycle leads to the formation of a plaque in the lawn of *M. smegmatis*. Since each plaque formed represents one mycobacterial cell in the original sample the assay can be used to enumerate the mycobacterial cultures (data reported as PFU ml^{-1} [10]). Antibiotic sensitivity/MIC experiments were carried out by adding rifampicin (5 µg ml^{-1}; RIF; Mast Diagnostics, UK). Phage will not replicate in RIF^S mycobacterial cells and no plaques will form at the end of the assay [10].

Phage amplification assay using TM4

The virucide used for TM4 was made from green tea (Gunpowder tea, Whittards of Chelsea, UK) using the method described by [16]. Briefly, the tea was prepared by adding sufficient RO water to the tea solids (7% w/v) and the samples were boiled for 10 min. The infusion was then filtered (Whatman Grade No. 2 Filter Paper, Whatman International Ltd.), autoclaved and stored at 4°C. The phage amplification assay was performed as described above, apart from 100 µl of tea infusion was used as the virucide and the virucide incubation time increased to 15 min.

Growth culture conditions

A method based on the Wayne model [3] was used to induce a non-replicating stationary phase in mycobacteria. Briefly the mycobacteria were grown in glass vials (12 ml volume) with a screw top lid filled to leave a head space ratio of 1:2 (air: liquid) and the tops sealed to finger tight (for oxygen-limited growth). The samples were then incubated at 37°C whilst shaking at 250 rpm. For the oxygenated cultures, cells were grown at 37°C with shaking (250 rpm) in the same vials with the lids loosely closed.

Phage attachment assay

The phage attachment assay was carried out as described by [17]. Briefly, the phage (approx. 10^7 PFU ml^{-1}) were added to each of the samples and incubated for up to 60 min to allow binding to their host cells. The samples were then centrifuged ($13000\times g$; 3 min) to remove the MAP cells and any bound phage. Unbound phage remaining in the supernatant were then titred.

Reversible inhibition of RNA synthesis using rifampicin

RIF-sensitive MAP cells, as determined by [18], were treated with an inhibitory concentration of RIF (5 µg ml^{-1}) for 24 h. To remove the antibiotic, cells were recovered by centrifugation ($13000\times g$; 3 min) and washed twice with MP, then resuspended in 1 ml of MP before further analysis performed.

Results

Detection of *M. smegmatis* cells using D29 and TM4

M. smegmatis was initially used as a fast-growing model organism for MAP. First *M. smegmatis* cells were grown to 1×10^7 CFU ml^{-1} either aerobically or under conditions where oxygen would become self-limiting as growth of the culture occurred (Wayne's model [3]) which took 10 d in a 37°C incubator shaking at 200 rpm. Each day, samples (100 µl) were removed for viable count determination and for enumeration using the phage amplification assay.

The results (Fig. 1A) show that there was no difference ($P>0.05$) in the number of *M. smegmatis* cells detected by culture (CFU ml^{-1}) or by the phage assay (PFU ml^{-1}) when they were cultured aerobically over the whole time series, demonstrating that the phage amplification enumeration assay compared well with traditional culture when used on aerobically grown *M. smegmatis* cells. However when the *M. smegmatis* was grown in oxygen limiting conditions, the PFU ml^{-1} values were almost one $\log_{10}$ lower than the CFU ml^{-1} value recorded after 10 d (Fig. 1A) showing that the phage were no longer able to detect the *M. smegmatis* cells efficiently when they were cultured under these conditions.

To determine whether the undetectable nature of the cells could be reversed, the *M. smegmatis* cells grown under oxygen limited conditions were diluted into fresh MP and incubated aerobically (shaking at 37°C at 200 rpm) and tested for 3 d and this was compared against the aerobically grown cells. At time point 0 approximately 10^4 PFU ml^{-1} of *M. smegmatis* cells were detected. After 1 d of aerobic incubation, the phage assay was able to detect nearly 10^7 PFU ml^{-1}. The number of cells detected in the culture transferred from the anaerobic growth conditions increased until after 3 d there was no difference ($P>0.05$) in PFU ml^{-1} values obtained for both cultures (Fig. 1B).

TM4 is broad spectrum mycobacteriophage that has been postulated to have the ability to infect non-replicating mycobacteria in the stationary phase of growth. Since the virucide used for D29 does not inactivate TM4 efficiently, tea infusions were tested for use as a virucide in the phage amplification assay as previously described [16]. The tea extract was shown to cause a 6-$\log_{10}$ destruction of TM4 within 15 min while having no adverse effect on the viability of mycobacteria tested (both MAP and *M. smegmatis*; Fig. S1). Using these extracts the *M. smegmatis* experiment was repeated using phage TM4 as well as phage D29.

The viable count of the *M. smegmatis* cultures grown aerobically or under oxygen limiting conditions were very similar (both approx. 1×10^4 CFU ml^{-1}; Fig 2). The results of the phage amplification assays showed that TM4 was able to infect *M. smegmatis* grown under both conditions, although the efficiency with which cells were detected was lower (only 1.58×10^2 PFU ml^{-1} detected in both samples; Fig. 2). In contrast D29 was not able to infect the cells grown under limiting oxygen conditions at all but for the cells growing in the presence of oxygen, the number of cells detected by D29 was not significantly different ($P>0.05$) from the viable count (1.6×10^4 CFU ml^{-1}). These results indicate that D29 is more efficient at infecting *M. smegmatis* cells grown under aerobic conditions than TM4 in this medium.

Detection of MAP cells using D29 and TM4

To determine whether the growth conditions affected the ability of phage D29 to infect a slow-growing mycobacterial species, this experiment was repeated using three strains of MAP (K10, DVL 453 and ATCC 19851). When cultures were grown using the Wayne's model conditions for 1 month, no MAP cells detected

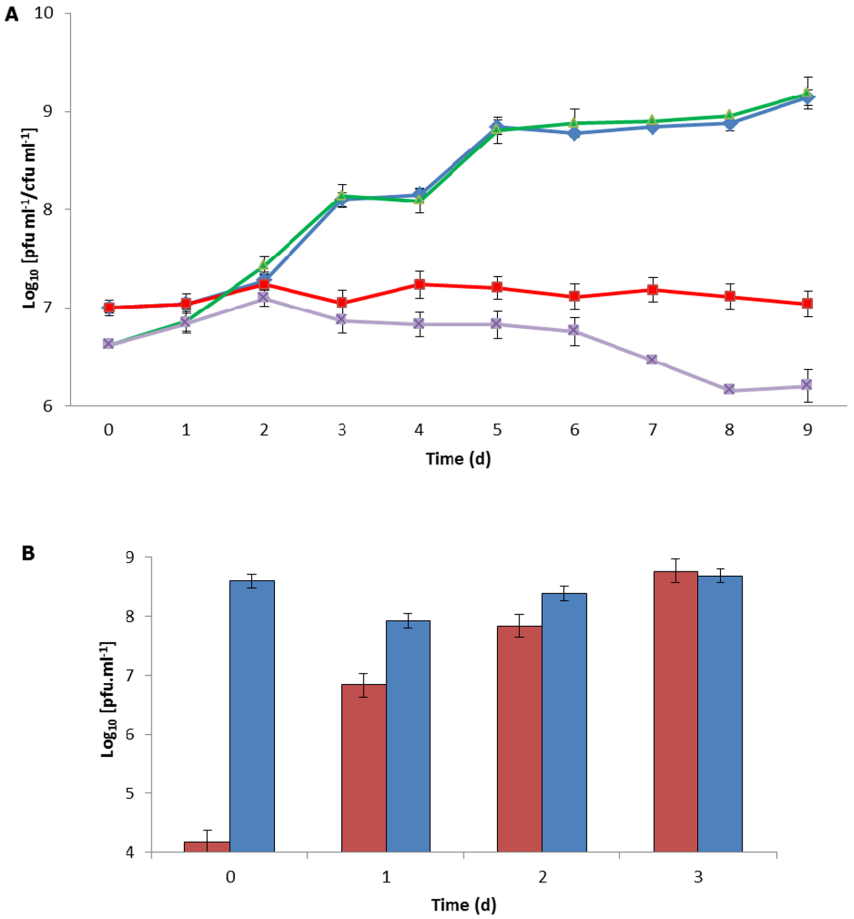


Figure 1. Comparison of *M. smegmatis* cells detected by phage and viable count under growth limiting and aerobic conditions. Panel A Graph showing the results of the phage assay (PFU ml^{-1}; green and purple) and viable count (CFU ml^{-1}; blue and red) for *M. smegmatis* cultured over a 10 d period under self-limiting oxygen conditions (red and purple) or under conditions were free oxygen exchange occurred (blue and green). T = 0 are values recorded for initial cultures before incubation. Error bars represent the standard deviations of the means of number of plaques recovered from the phage assay performed in triplicate. Panel B Graph showing the number of *M. smegmatis* cells detected using the phage amplification assay (PFU ml^{-1}). Prior to dilution into fresh medium the *M. smegmatis* cells were either grown under self-limited oxygen conditions (red bars) or aerobic conditions (blue bars). Samples were taken from the fresh cultures over a 3 d period. Error bars represent the standard deviations of the means of number of plaques recovered from the phage assay performed in triplicate.
doi:10.1371/journal.pone.0106690.g001

using phage D29 although growth was visible in the cultures. Cells from this phage-undetectable culture were then inoculated into fresh MP (100 µl cells into 9.9 ml MP) without Mycobactin-J. This was to limit growth so that recovery of cells from the uninfectable state would be more apparent.

After one day incubation with aeration, only cells in the DVL 453 strain were detectable using the phage amplification assay (1.5×10^1 PFU ml^{-1}; Fig. 3). On day 2, a 2–3 $\log_{10}$ increase in the number of cells detected by the phage assay was seen for each strain of MAP tested. After 7 days of aerated incubation, the number of MAP cells detected using D29 in the phage amplification assay was between 10^2 to 10^5 PFU ml^{-1}. As there was no Mycobactin-J added to the cultures this suggest that there was an increase in infectivity rather than an increase in cell number.

When the experiment was repeated with phage TM4 the results show that for cells grown under oxygen limiting conditions 1.8×10^3 PFU ml^{-1} MAP were detected whereas, once again, D29 did not detect any MAP cells (Fig. 4). As before, when these cells were exposed to air for 9 d, phage D29 was able to detect significantly more MAP cells (1.5 $\log_{10}$ PFU ml^{-1}; $P<0.01$) compared to TM4, confirming that phage D29 is able to infect the mycobacteria more efficiently than TM4 when the cells are well aerated in this media.

Attachment of phage D29 to non-detectable MAP cells

To investigate what might be preventing successful D29 phage infection, a phage attachment assay was performed. MAP cells (1×10^5 PFU ml^{-1}) were grown for 1 month under oxygen limiting conditions (using Wayne's model) and aerobically. The samples were then mixed with phage D29 (1×10^7 PFU ml^{-1}) for 0, 30 and 60 min to allow attachment of phage to the cells. Infected cells/phage attached to cells were then removed from the culture by centrifugation and the number of phage remaining in the supernatant titred. The results show that there was an approximate 20% drop in the number of free phage particles present in the culture supernatant for both cell cultures after 60 min of incubation with the phage (Fig. 5), and there was no statistical difference ($P>0.05$) in the level of attachment of phage to the cells in the two cultures. When bacteriophage were added to media without cells, there was no reduction in phage titre, showing that the loss of phage from the supernatant was due to the attachment

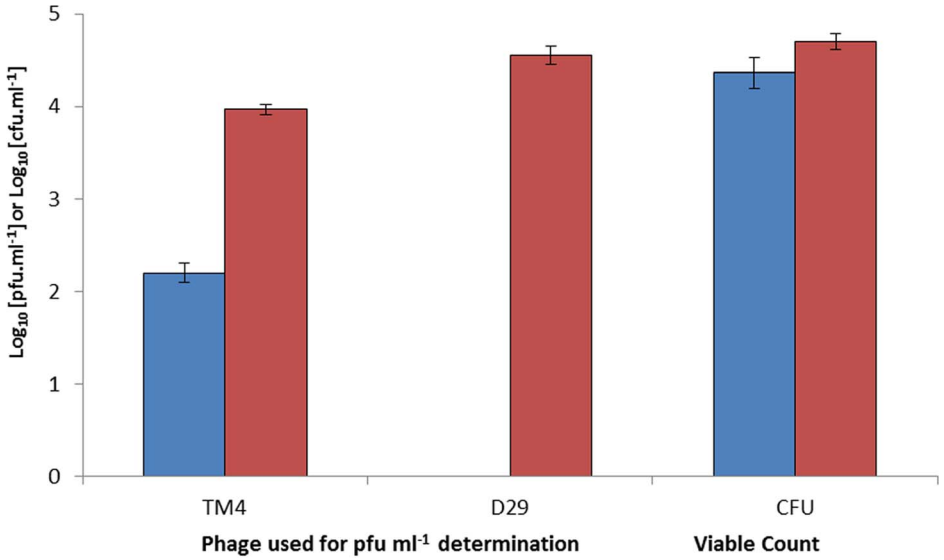


Figure 2. Detection of *M. smegmatis* using D29 and TM4. Graph showing the number of plaques recovered following the phage assay when using phage TM4 and D29. The *M. smegmatis* cells tested were either grown with limiting oxygen (blue bars) or after these cells had been grown with aeration (red bars). In addition to the phage assay the viable count (CFU ml^{-1}) of both cultures was determined. Error bars represent the standard deviations of the means of number of plaques and colonies recovered from the phage assay and viable count, respectively, performed in triplicate.
doi:10.1371/journal.pone.0106690.g002

of phage to the cells. This result suggests that the D29 receptors are not altered or lost when the mycobacteria are grown under oxygen limiting conditions, but productive infection is blocked.

Role of RNA synthesis inhibition on phage infection

Antibiotics that block RNA synthesis can be used to determine if *de novo* protein synthesis is required for an adaptive event to occur. However, before determining whether differences during dormancy were the reason for the inhibition of phage infection, the effect of the antibiotic on resuscitation of hosts was investigated. First the sensitivity of the MAP K10 cells to RIF was determined using the *FASTPlaque*TB^{TM} Response protocol [10]. To do this MAP K10 cells were treated with an inhibitory concentration of RIF (5 μg ml−1) and the phage assay was then performed. The results showed that the MAP cells were sensitive to RIF since plaque formation was inhibited completely. To then determine whether the RIF inhibition of RNA polymerase was reversible for MAP, the antibiotic was removed from RIF-treated cells by centrifugation (13000×g; 3 min) and washing twice with MP. In this case plaques were formed showing that productive phage replication is RIF sensitive and that RIF inhibition is reversible and the number of plaques formed were not significantly different ($P>0.05$) to the numbers before RIF treatment (Fig. S2).

To determine whether D29 phage infection was inhibited by *de novo* protein synthesis, antibiotics were used to transiently inhibit RNA synthesis in the host mycobacteria. MAP cells were grown

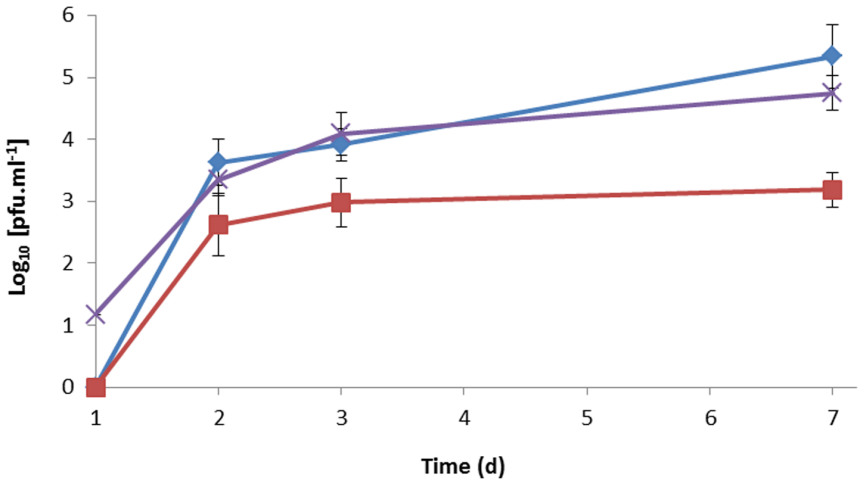


Figure 3. Recovery of phage D29 infectivity by three strains of MAP. Graph showing the number of MAP cells detected using the RapidMAP assay (PFU ml^{-1}). Prior to dilution into fresh medium the MAP cells were either grown under self-limiting oxygen conditions and then samples were taken from the fresh cultures over a 7 d period. The three strains of MAP used were K10 (blue), DVL 453 (purple) and ATCC 19851 (red). Error bars represent the standard deviations of the means of number of plaques recovered from the phage assay performed in triplicate.
doi:10.1371/journal.pone.0106690.g003

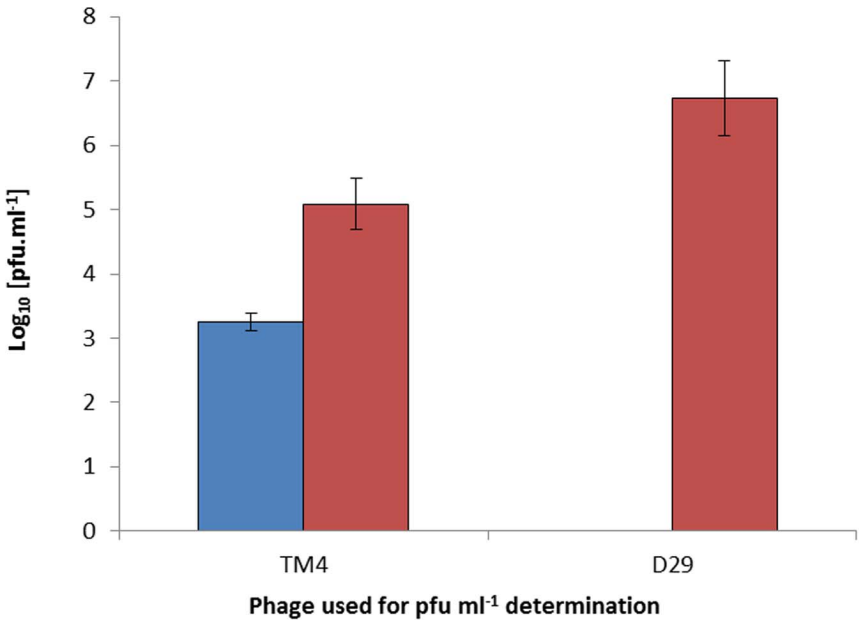


Figure 4. Difference in infectivity of MAP cells by D29 and TM4. Graph showing the number of plaques recovered following the phage assay when using phage TM4 and D29. The MAP cells tested were either grown with limiting oxygen (blue bars) or after these cells had been exposure to air for 9 days (red bars). Error bars represent the standard deviations of the means of number of plaques recovered from the phage assay performed in triplicate.
doi:10.1371/journal.pone.0106690.g004

for 1 month under oxygen-limiting conditions to induce the uninfectable state, and then treated with RIF. After one day aeration, there was a significant ($P<0.01$) three-$\log_{10}$ increase in the number of MAP cells detected in the sample that was not treated with RIF (Fig. 6). However after the antibiotic was washed out of the RIF-treated cells using the method described above, no MAP cells were detected in the RIF-treated sample. This result suggests that there is a requirement for *de novo* gene expression for conversion of the MAP cells back into and infectable state.

Discussion

The phage amplification assay is a powerful tool that can detect, enumerate and determine the antibiotic sensitivity of mycobacteria. However, a lack of information about phage-host interactions may lead to inaccurate interpretation of results. Phage D29 was found to be unable to infect both MAP and *M. smegmatis* cells when they were induced into a non-growing phase, when oxygen became self-limiting in the growth tubes, as described by [3].

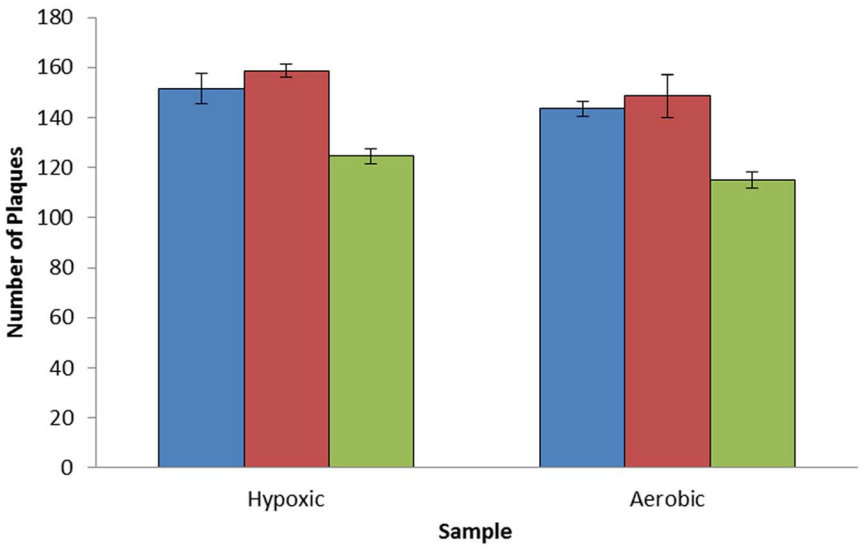


Figure 5. Effect of stationary phase bacteria on the attachment of phage D29 to MAP cells. Graph showing the number of unbound phage particles to MAP cells that are infectable (aerobic) and uninfectable (hypoxic) after 0 min (blue bars), 30 min (red bars) and 60 min (green bars). Error bars represent the standard deviations of the means of number of bacteriophage detected after each time point in triplicate.
doi:10.1371/journal.pone.0106690.g005

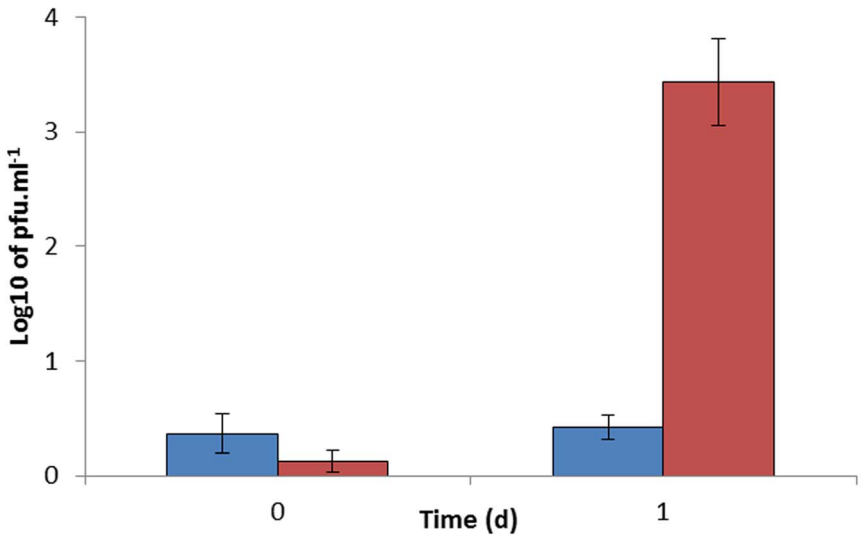


Figure 6. Role of RNA synthesis in recovery of phage-sensitivity. Graph showing the number of MAP cells detected by the RapidMAP assay, after uninfectable MAP cells were treated with RIF (blue bars) and without RIF (red bars) before exposure to oxygen (t – 0) and after exposure to oxygen (t – 1). Error bars represent the standard deviations of the means of number of plaques recovered from the phage assay performed in triplicate.
doi:10.1371/journal.pone.0106690.g006

However, when the cells were reintroduced to oxygen, the ability of the phage to infect the cells was restored almost completely although the time required to achieve this was different for the two organisms tested; three days for *M. smegmatis* and over one week for MAP. This result probably reflects the very different natural growth rates of these two bacterial species.

M. smegmatis, *M. bovis* and *M. tuberculosis* have all been reported to have the ability to enter a 'non-replicating' stationary phase during hypoxic stress [2,3,19]. Hypoxia is predicted to be a key host-induced stress, limiting growth of pathogenic mycobacteria *in vivo*. Many studies have indicated that *M. tuberculosis* adapts to oxygen limitation by entering into a metabolically altered state while awaiting the opportunity to reactivate [20]. Interestingly, using D29 to perform the phage amplification assay, we have been able to sensitively detect MAP in white blood cells isolated from the blood of naturally infected animals [9]. Our discovery concerning the effect of hypoxia on the efficiency of D29 infection suggests that these cells recovered from clinical samples are not in a state of hypoxic stress and perhaps then are not dormant. Whittington *et al*. [4] presented evidence that MAP present on grass could enter a dormant state similar to that described for *M. tuberculosis* and suggested that this may facilitate the survival of this organism in the environment, however when present on the surface of grass it would not be anticipated that oxygen was limiting for these cells.

It is known that adsorption of D29 to *M. tuberculosis* is more efficient when the bacteria are in exponential phase of growth [21]. The reduction in the adsorption efficiency was thought to be due to structural changes on the cell wall of the host, such as cell wall thickening due to the accumulation of alpha-crystallin chaperone protein [3,5], which occur when they are not in the exponential growth phase, and this in turn may affect the accessibility of D29 to specific phage receptor sites [21]. Recently some MAP strains have been reported to undergo morphological changes during nutrient starvation [22] and cell wall thickening could prevent phage D29 from attaching to the cell surface and binding its receptor. However in this study the phage attachment assay suggests there was no change in the ability of the phage to bind to MAP cells, and therefore there this suggests that simple changes in the cell wall structure alone do not explain the inhibition of productive phage infection.

When MAP cells induced into the hypoxic non-infectable state were treated with RIF to prevent *de novo* protein synthesis, the cells were unable to fully revert to an infectable state until the RIF was washed out of the cells, suggesting that there is a requirement of RNA synthesis and *de novo* gene expression for the transition of the cells to active aerobic growth. Researchers have found that a homologue of the DNA binding-like protein (Dps) in the *M. avium* genome, which was first identified in *M. smegmatis* and confers protection by binding to DNA during nutritional and oxidative stress in other bacteria [4]. Dps has been shown to confer resistance to bacteriophage that infect *E. coli*. Dps was found to be present in phage sensitive *E. coli* cells and was thought to be the reason for the phage resistance [23]. Thus resistance to D29 phage infection may be due to an accumulation of proteins such as Dps which would bind to the replicating phage DNA and prevent productive phage replication and it may require the expression of proteases or other regulatory proteins to remove the accumulated Dps from the cells.

In contrast to D29, bacteriophage TM4 has been reported to be able to infect stationary phase mycobacteria [24,25]. It has been postulated that this difference is due to a peptidoglycan hydrolase motif found on the tape measure protein of TM4 that is not present on the tail of D29. This motif is thought to act in a similar way to resuscitation protein factors (Rpfs) which can induce stationary phase mycobacteria cells into an active growth state [26] by generating a signal that mimics the Rpfs leading to mycobacteria resuscitation, and therefore productive TM4 replication. It was also notable that TM4 infection of both *M. smegmatis* and MAP was not as efficient as that achieved by phage D29 when aerated cultures were used. However this may simply reflect the fact that the growth media used for the phage amplification assay was optimised for D29 infection and contains 2 mM $CaCl_2$, which has been reported to inhibit TM4 infection of *M. smegmatis* and *M. bovis* BCG cells [27].

The results in this study demonstrate that bacteriophage TM4 and D29 can be used as a tool to identify when MAP adapts to hypoxic stress. As tools, they could be used to determine how many other stress conditions, such as UV and pH [4] also induce this response and whether different environmental conditions induce difference responses in the host that can be detected by changes in efficiency of phage infection. It would be interesting to identify genes required to be expressed to allow transition to occur as way of understanding the basis of this adaptive response and these phage would provide a good tool to screen for such genes.

Supporting Information

Figure S1 The ability of tea and FAS to inactivate phage TM4 and D29. Graph showing the number of phage particles remaining after treatment with green tea (Tea) and ferrous ammonium sulphate (FAS). Error bars represent the standard deviations of the mean number plaques recovered from the phage titre.
(TIF)

Figure S2 The effect of washing out RIF on MAP cell detectability with the phage assay. Graph showing whether phage D29 can infect RIF^s MAP cells exposed to RIF and when RIF is washed away. Error bars represent the standard deviations of the mean number plaques recovered from the phage amplification assay.
(TIF)

Author Contributions

Conceived and designed the experiments: BMCS ZEG JNH CEDR. Performed the experiments: BMCS ZEG JNH CEDR. Analyzed the data: BMCS ZEG JNH CEDR. Contributed to the writing of the manuscript: BMCS ZEG JNH CEDR.

References

1. Zimmer K, Drager KG, Klawonn W, Hess RG (1999) Contribution to the diagnosis of Johne's disease in cattle. Comparative studies on the validity of Ziehl-Neelsen staining, faecal culture and a commercially available DNA-Probe (R) test in detecting Mycobacterium paratuberculosis in faeces from cattle. Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health 46: 137–140.
2. Dick T, Lee BH, Murugasu-Oei B (1998) Oxygen depletion induced dormancy in Mycobacterium smegmatis. FEMS Microbiol Lett 163: 159–164.
3. Wayne LG, Hayes LG (1996) An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence. Infect Immun 64: 2062–2069.
4. Whittington RJ, Marshall DJ, Nicholls PJ, Marsh AB, Reddacliff LA (2004) Survival and dormancy of Mycobacterium avium subsp paratuberculosis in the environment. Applied and Environmental Microbiology 70: 2989–3004.
5. Cunningham AF, Spreadbury CL (1998) Mycobacterial stationary phase induced by low oxygen tension: Cell wall thickening and localization of the 16-kilodalton alpha-crystallin homology. Journal of Bacteriology 180: 801–808.
6. Gumber S, Taylor DL, Marsh IB, Whittington RJ (2009) Growth pattern and partial proteome of Mycobacterium avium subsp. paratuberculosis during the stress response to hypoxia and nutrient starvation. Vet Microbiol 133: 344–357.
7. Rybniker J, Kramme S, Small PL (2006) Host range of 14 mycobacteriophages in Mycobacterium ulcerans and seven other mycobacteria including Mycobacterium tuberculosis - application for identification and susceptibility testing. Journal of Medical Microbiology 55: 37–42.
8. Hatfull GF (2010) Mycobacteriophages: genes and genomes. Annu Rev Microbiol 64: 331–356.
9. Hatfull GF (2012) The secret lives of mycobacteriophages. Adv Virus Res 82: 179–288.
10. Rees CR, Botsaris G (2012) The Use of Phage for Detection, Antibiotic Sensitivity Testing and Enumeration. In: Cardona P-J, editor. Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis. Rijeka: InTech. pp. 293–306.
11. Swift BM, Denton EJ, Mahendran SA, Huxley JN, Rees CE (2013) Development of a rapid phage-based method for the detection of viable Mycobacterium avium subsp. paratuberculosis in blood within 48 h. J Microbiol Methods 94: 175–179.
12. Botsaris G (2010) Development and Evaluation of a Rapid Phage-PCR Assay to Detect Mycobacterium avium subsp. paratuberculosis in Dairy Products. Leics: University of Nottingham. 297 p.
13. Botsaris G, Slana I, Liapi M, Dodd C, Economides C, et al. (2010) Rapid detection methods for viable Mycobacterium avium subspecies paratuberculosis in milk and cheese. International Journal of Food Microbiology 141: S87–S90.
14. Stanley EC, Mole RJ, Smith RJ, Glenn SM, Barer MR, et al. (2007) Development of a new, combined rapid method using phage and PCR for detection and identification of viable Mycobacterium paratuberculosis bacteria within 48 hours. Applied and Environmental Microbiology 73: 1851–1857.
15. Foddai A, Elliott CT, Grant IR (2009) Optimization of a Phage Amplification Assay To Permit Accurate Enumeration of Viable Mycobacterium avium subsp paratuberculosis Cells. Applied and Environmental Microbiology 75: 3896–3902.
16. de Siqueira RS, Dodd CER, Rees CED (2006) Evaluation of the natural virucidal activity of teas for use in the phage amplification assay. International Journal of Food Microbiology 111: 259–262.
17. Spears PA, Suyemoto MM, Palermo AM, Horton JR, Hamrick TS, et al. (2008) A Listeria monocytogenes mutant defective in bacteriophage attachment is attenuated in orally inoculated mice and impaired in enterocyte intracellular growth. Infection and Immunity 76: 4046–4054.
18. Albert H, Trollip A, Seaman I, Mole RJ (2004) Simple, phage-based (FASTPlaque) technology to determine rifampicin resistance of Mycobacterium tuberculosis directly from sputum. International Journal of Tuberculosis and Lung Disease 8: 1114–1119.
19. Hutter B, Dick T (1999) Up-regulation of narX, encoding a putative 'fused nitrate reductase' in anaerobic dormant Mycobacterium bovis BCG. FEMS Microbiol Lett 178: 63–69.
20. Rustad TR, Sherrid AM, Minch KJ, Sherman DR (2009) Hypoxia: a window into Mycobacterium tuberculosis latency. Cellular Microbiology 11: 1151–1159.
21. David HL, Clavel S, Clement F (1980) Adsorption and Growth of the Bacteriophage-D29 in Selected Mycobacteria. Annales De Virologie 131: 167-&.
22. Lamont EA, Bannantine JP, Armien A, Ariyakumar DS, Sreevatsan S (2012) Identification and Characterization of a Spore-Like Morphotype in Chronically Starved Mycobacterium avium Subsp Paratuberculosis Cultures. Plos One 7.
23. Lacqua A, Wanner O, Colangelo T, Martinotti MG, Landini P (2006) Emergence of biofilm-forming subpopulations upon exposure of Escherichia coli to environmental bacteriophages. Applied and Environmental Microbiology 72: 956–959.
24. Foley-Thomas EM, Whipple DL, Bermudez LE, Barletta RG (1995) Phage Infection, Transfection and Transformation of Mycobacterium-Avium Complex and Mycobacterium-Paratuberculosis. Microbiology-Uk 141: 1173–1181.
25. Dusthackeer A, Kumar V, Subbian S, Sivaramakrishnan G, Zhu GF, et al. (2008) Construction and evaluation of luciferase reporter phages for the detection of active and non-replicating tubercle bacilli. Journal of Microbiological Methods 73: 18–25.
26. Piuri M, Hatfull GF (2006) A peptidoglycan hydrolase motif within the mycobacteriophage TM4 tape measure protein promotes efficient infection of stationary phase cells. Molecular Microbiology 62: 1569–1585.
27. Fullner KJ, Hatfull GF (1997) Mycobacteriophage L5 infection of Mycobacterium bovis BCG: implications for phage genetics in the slow-growing mycobacteria. Molecular Microbiology 26: 755–766.

Manuscript Details

Manuscript number	FM_2017_830
Title	Survival of Mycobacterium avium subspecies paratuberculosis in retail pasteurised milk
Article type	Research Paper

Abstract

A survey of retail purchased semi-skimmed pasteurised milk (n=368) for Mycobacterium avium subspecies paratuberculosis (MAP) was conducted between May 2014 and June 2015 across the midlands of England using the Phage-PCR assay. Overall, 10.3 % of the total samples collected contained viable MAP cells, confirming that pasteurisation is not capable of fully eliminating human exposure to viable MAP through milk. Comparison of the results gained using the Phage-PCR method with the results of surveys using either culture or direct PCR suggest that the phage-PCR method is able to detect lower numbers of cells, resulting in an increase in the number of MAP-positive samples detected. Comparison of viable count and levels of MAP detected in bulk milk samples suggest that MAP is not primarily introduced into the milk by faecal contamination but rather are shed directly into the milk within the udder. In addition results detected an asymmetric distribution of MAP cells exists in the milk matrix prior to white cell lysis, indicating that the MAP cells in naturally contaminated milk are clustered together and may primarily be located within somatic cells. These latter two results lead to the hypothesis that intracellular MAP within the somatic cells may be protected against heat inactivation during pasteurisation.

Keywords	paratuberculosis; Johne’s disease; Crohn’s disease; pasteurised milk;

Corresponding Author	Benjamin Swift
Corresponding Author's Institution	University of Nottingham
Order of Authors	Zara Gerrard, Benjamin Swift, George Botsaris, Ross Davidson, Mike Hutchings, Jonathan Huxley, Catherine Rees

Submission Files Included in this PDF

File Name [File Type]

Cover Letter Milk Paper.docx [Cover Letter]

Highlights.docx [Highlights]

Paper Final.docx [Manuscript File]

Figure 1.jpg [Figure]

Figure 2.jpg [Figure]

Figure 3.jpg [Figure]

Figure 4.jpg [Figure]

Figure 1 Caption.docx [Figure]

Figure 2 caption.docx [Figure]

Figure 3 caption.docx [Figure]

Figure 4 caption.docx [Figure]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

School of Veterinary Medicine and Science

Sutton Bonington Campus
Loughborough
LE12 5RD
Tel +44 (0)115 951 6167
Fax +44 (0)115 951 6162
http://www.nottingham.ac.uk/vet

Ben Swift

e-mail: benjamin.swift@nottingham.ac.uk

The Editor

Food Microbiology

26th September 2017

re: Survival of Mycobacterium avium subspecies paratuberculosis in retail pasteurised milk

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a very slow growing organism that causes Johne's disease in cattle and other ruminants and has been linked with Crohn's disease in humans. The ability to detect MAP quickly is thought to be vital in control of the disease throughout a herd. We have previously described a rapid bacteriophage-based method that can detect MAP in the milk of cows naturally infected with Johne's disease within 48 h (Stanley et al. 2007; Botsaris et al. 2010). Here we describe the use of the phage-PCR method to detect viable MAP in a survey of 368 retail pasteurised milk samples and to demonstrate potential mechanisms of pasteurisation survival.

We have previously described rapid bacteriophage-based methods that can rapidly detect and enumerate mycobacteria in clinical samples of milk and blood (Stanley et al., 2007. Appl. Environ. Microbiol. 73:1851-1857; Swift et al., 2013. J. Microbiol. Methods. 3: 175-179). This method exploits a broad host range bacteriophage (D29) which infects all species of mycobacteria tested to date. Our work with other Johne's disease has shown that the phage assay is very sensitive and is capable of detecting very low levels of MAP in a milk sample.

Our use of the phage assay and its application on a very important food issue as well as our further investigation into MAP mechanism of the survival demonstrate that MAP is capable of entering the human food chain and our ability to detect low levels could help inform the debate of milk processing to reduce the burden on MAP in retail milk samples and we feel publication in Food Microbiology would be the ideal platform to disseminate our impactful research.

We hope that you find this paper suitable for publication.

Yours sincerely

Ben Swift
BSc (Hons), MRes, PhD

Highlights

- Survey of retail pasteurised milk for presence of *M. paratuberculosis*
- Use of phage-PCR to rapidly and sensitively detect *M. paratuberculosis* in milk
- Presence of viable *M. paratuberculosis* in 10 % of samples
- Intracellular *M. paratuberculosis* may aid their survival during pasteurisation

Survival of *Mycobacterium avium* subspecies *paratuberculosis* in retail pasteurised milk

Zara E. Gerrard [a,b], Benjamin M. C. Swift[a*], George Botsaris[c], Ross S. Davidson[b], Michael R. Hutchings[b], Jonathon N. Huxley[a], Catherine E.D. Rees[a]

[a]University of Nottingham, Sutton Bonington Campus, College Road, Leicestershire, LE12 5RD

[b]SRUC, West Mains Road, Edinburgh EH9 3JG

[c]Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus

*Corresponding author

Email: benjamin.swift@nottingham.ac.uk

Abstract

A survey of retail purchased semi-skimmed pasteurised milk (*n*=368) for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was conducted between May 2014 and June 2015 across the midlands of England using the Phage-PCR assay. Overall, 10.3 % of the total samples collected contained viable MAP cells, confirming that pasteurisation is not capable of fully eliminating human exposure to viable MAP through milk. Comparison of the results gained using the Phage-PCR method with the results of surveys using either culture or direct PCR suggest that the phage-PCR method is able to detect lower numbers of cells, resulting in an increase in the number of MAP-positive samples detected. Comparison of viable count and levels of MAP detected in bulk milk samples suggest that MAP is not primarily introduced into the milk by faecal contamination but rather are shed directly into the milk within the udder. In addition results detected an asymmetric distribution of MAP cells exists in the milk matrix prior to white cell lysis, indicating that the MAP cells in naturally contaminated milk are clustered together and may primarily be located within somatic cells. These latter two results lead to the hypothesis that intracellular MAP within the somatic cells may be protected against heat inactivation during pasteurisation.

Keywords

Paratuberculosis; Johne's disease; Crohn's disease; pasteurised milk;

1.0 Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic wasting disease of cattle and other ruminants, characterised by a reduction in milk yield, severe diarrhoea, weight loss and intermandibular oedema (commonly referred to as bottle jaw). In addition to causing a significant health impact on the national herd, this disease has a significant economic impact on the dairy industry as a whole. MAP is an extremely slow growing bacterium, taking up to 8-12 weeks to grow in liquid culture media, which makes detection by traditional culture methods problematic (Sweeney et al., 2012). MAP is also implicated in the development of Crohn's disease (CD), an inflammatory bowel condition of humans with similar aetiology to JD (Bull et al., 2003; Feller et al., 2007; Rhodes et al., 2014), and therefore it is recommended that limiting human exposure would be sensible on a precautionary principle (NACMCF, 2010).

Cattle infected with MAP can shed substantial levels of the bacteria into their milk, faeces and semen (Antognoli et al., 2008), with milk being highlighted as a key transmission vehicle for human exposure to MAP, making the efficiency of pasteurisation an important factor in controlling transmission (Stabel and Lambertz, 2004). Although many studies have shown that the conditions used for pasteurisation are sufficient to inactivate MAP, some studies have shown that both low-temperature long-time (LTLT) and high-temperature short-time (HTST) pasteurisation did not totally inactivate MAP when added to samples at greater than 1×10^4 cfu ml^{-1} (Chiodini and Hermontaylor, 1993; Grant et al., 1996). This finding led to many dairy processing centres to adjust the holding time of HTST pasteurisation from 15 s (the legal minimum in the UK), to 25 s to increase the likelihood of totally inactivating MAP. Despite this, MAP has been reported to survive the pasteurisation process and has been detected by culture in a number of surveys of retail pasteurised milk with a prevalence ranging between 1.7 to 6.7 % of samples tested (Ayele et al., 2005; Grant et al., 2002; Grant et al., 2002; Paolicchi et al., 2012).

The detection of MAP has been historically difficult due to short-falls in standard culture and PCR-based detection methods. Milk culture requires chemical decontamination to eliminate fast growing bacteria, but this also causes a 1-2 $\log_{10}$ drop in viable MAP cells, which in turn can decrease the chance of successful culture (Dundee et al., 2001). For pasteurised dairy products, PCR-based detection is less useful since it will detect any MAP DNA present in the sample, whether this is from viable bacteria or from cells that have been inactivated by the heat treatment. The bacteriophage amplification assay coupled with PCR (phage-PCR) has been developed to rapidly and sensitively detect viable MAP as an alternative to culture-based methods (Swift and Rees, 2013). This assay uses Mycobacteriophage D29 to infect any mycobacteria present in a milk sample, and any viable mycobacteria present will be indicated by the growth of the bacteriophage. PCR can then be used to confirm the identity of the mycobacteria detected (Stanley et al., 2007). The phage assay is faster than culture as results can be gained within 48 h, as well as being more sensitive than culture as there is no need for chemical decontamination. The phage assay has been successfully used to detect viable MAP in raw milk (Botsaris et al., 2010; Stanley et al., 2007), powdered infant formula (Botsaris et al., 2016), cheese (Botsaris et al., 2010) and blood of infected animals (Swift et al., 2013). In this study we used the phage-PCR assay to rapidly estimate the prevalence of MAP in pasteurised milk. In addition experiments were carried out to try and establish how MAP enters the milk and where the bacteria are located in naturally contaminated milk, and the pattern of results led to a hypotheses of how MAP survives pasteurisation.

2.0 Material and Methods

2.1 Collection of pasteurised milk samples

Three-hundred and eighty-six semi-skimmed milk (1.7 % fat) samples were collected at four time points (May 2014, November 2014, January 2015, and June 2015) using volunteers from the University

of Nottingham Sutton Bonington campus; this type of milk was chosen as it was the most popular purchased by the volunteers providing the samples. The milk had been purchased from either retail suppliers or doorstop providers primarily from three UK counties (Nottinghamshire, Derbyshire, and Leicestershire). Volunteers were provided with a sterile 50 ml tube and detailed instructions on how to take the sample, including specifying that it must be taken from a previously unopened carton (carton size not specified) that was shaken to uniformly mix the contents before samples were taken and that the 50 ml sample should be delivered to the laboratory within two days of purchase. Details of the date of pasteurisation and retail supplier were also provided, but are not discussed here.

2.2 Total viable count

The viable count was performed using milk count agar according to the National Standard Method D2 (Health Protection Agency, UK). Serial dilutions of the milk sample were prepared and samples (100 µl) spread plated before plates were incubated aerobically at 30 °C for 3 d for enumeration.

2.3 Phage-PCR assay

Briefly, 25 ml of milk was centrifuged at 2,500 *x g* for 15 min to separate the pellet, milk and cream layers. The upper two layers were removed, and the pellet resuspended in 3 ml Modified Middlebrook 7H9 media plus (MP; Middlebrook 7H9 broth supplemented with OADC (oleic acid, bovine albumin, dextrose and catalase; Becton Dickinson, UK), NOA (nystatin, oxacillin and aztreonam Mole et al., 2007), and 2 mM $CaCl_2$) and centrifuged at 2,500 *x g* for 10 min. The resulting pellet was finally resuspended in 1 ml of MP and mycobacteriophage D29 (100 µl of 10^9 pfu ml^{-1} phage suspension) added to the sample. After incubation at 37 °C for 1 h a virucide (100 µl of 10 mM ferrous ammonium sulphate) was added to destroy any exogenous phage, and the sample finally plated with *M. smegmatis* (1 ml of 10^8 cfu ml^{-1} culture grown in MP) and 6 ml of Middlebrook 7H10 agar. Plates were then incubated for 24 h and plaques enumerated. DNA was extracted from plaque agar using Zymoclean Gel DNA Recovery kit (Zymo Research) and the presence of MAP DNA detected by

amplification of MAP-specific IS*900* region using a nested-PCR (Bull et al., 2003). Only samples that produced a positive IS*900*-PCR result were classified as containing viable MAP cells.

2.4 Investigation of location of MAP cells in milk

One litre of raw milk was obtained from a local farm's bulk tank and split into 20 x 50 ml samples. Ten of these were processed as described in Section 2.3. For the other 10 samples, after the sample was resuspended in 2 ml MP which lyses any somatic cells, the sample was split into two portions and each one tested separately using the bacteriophage assay described in section 2.3.

2.5 Analysis

Pfu and cfu counts were compared using Pearson correlation and MAP status (positive or negative) with TVC by performing a t-test in IBM SPSS Statistics 22 (SPSS Inc., Chicago, USA). Further analysis of the correlation between the pfu counts in the paired split bulk tank milk samples was conducted in Microsoft Excel 2010. Details of the statistical methods used to determine the complete spatial randomness (CSR) of cells in bulk tank milk are given in the text.

3.0 Results and Discussions

3.1 Detection of MAP in pasteurised milk

The phage-PCR assay detected viable MAP in 10.3 % (37/368) of the pasteurised milk samples collected as defined by the presence of plaques which gave a positive IS*900*-PCR result (see Figs. 1 & 2). Figure 1 shows the number of MAP-positive samples recorded at each of the four sampling time points (approximately 90 samples at each time point). The results show that although the overall average percentage of MAP-positive samples detected was 10.3 %, there was a large variation in the number of positive samples detected at each time point (range 1-27 %), but there was no obvious seasonal pattern. This is consistent with the results of Grant et al. (2002) who detected variable levels

of MAP in retail pasteurised milk using a direct PCR-method (average 10 %, range 0-27 %; Grant et al., 2002a).

Since each plaque arising from the phage-PCR method represents detection of one viable MAP cell, counting the plaques allows enumeration of MAP as well as absolute detection. Figure 2 shows the distribution of plaque numbers detected in MAP-positive samples collected throughout the survey. Only 1.1 % of these contained more than 10 detectable MAP cells (range 10-32). It is known that chemical decontamination methods reduce the number of MAP cells detectable by culture by least 1 $\log_{10}$ giving a low limit of detection (Bradner et al., 2013). Therefore it is unlikely that samples that contained less than 10 MAP cells per ml would give a culture-positive result in previous studies. On this basis, given that other published surveys of retail pasteurised milk generally report levels of detection in the 1-2 % range, our results are in line with previous culture-based studies.

Direct PCR detection of MAP in milk is believed to be more sensitive than culture with a detection limit approximately 2 cfu per sample (Logar et al., 2012). In this study, 3.5 % of MAP-positive samples (1.1 % >10 plus 2.4 % 3-9) contained more than 2 cells and therefore would theoretically be detectable by direct PCR. Other published surveys of retail pasteurised milk sample by direct PCR have reported detection of between 7 and 12 % of MAP-positive samples (Grant et al., 2002b), so again our results are within the range predicted by the published literature. However it must be noted that direct PCR-methods used to survey pasteurised milk do not discriminate between viable cells and cells that have been inactivated by pasteurisation, and therefore these results would also include samples where inactivated cells were detected, perhaps explaining the higher prevalence detected in some of these direct PCR studies. The largest number of samples contained 1-2 detectable MAP cells (6.8 %). Given that these samples contained fewer cells than the reported limit of detection of either culture or PCR-based detection, this result suggests that the level of MAP present would not be detected by either of these methods and that the phage-PCR method is a more sensitive technique.

Commercial pasteurisation has been shown by many surveys of retail milk to not be sufficient to inactivate all MAP cells present in raw milk. Although determinations of D-values indicated MAP may survive HTST pasteurisation when the initial organism concentration is greater than 1 x 10^4 cells ml^{-1} (Grant et al., 1996), many other studies have shown that the cells are not intrinsically resistant to heat and pasteurisation can result in a seven-fold reduction in viable MAP (Rademaker et al., 2007). However Grant et al. (1996) has previously reported that after an initial rapid drop in viable cell number, a 'long tail' in inactivation curves is seen whereby low numbers of viable MAP cells in a sample survive, even after heating for an extended period of time. This type of kinetics indicates that there is a heat-resistant sub-population, and would be consistent with our observation that the largest number of samples contained only 1-2 detectable MAP cells. One explanation for this phenomenon could be if the MAP cells were internalised within somatic cells which provided some protection against heat inactivation.

3.2 Evidence that MAP in milk is primarily intracellular

3.2.1 Comparison of TVC and Phage-PCR

It has been well established that low level faecal contamination of milk can occur during routine milking practises, therefore providing a route of entry for MAP into raw milk. To provide evidence that the MAP detected by the Phage-PCR assay represent cells that are shed directly into the milk rather than being introduced by faecal contamination, a comparison of the total viable count (TVC) of raw milk bulk tank samples and levels of MAP detected using the phage-PCR assay was undertaken. The samples were collected from 225 separate farms as previously described by Botsaris et al. (2013). Since the phage D29 has a very broad host range the phage assay results (plaque number) report on the total number of viable mycobacteria present in a sample. Figure 3a shows a comparison of the TVC results for all samples that were found to contain mycobacteria (plaque-positive samples; n = 218), and these samples were then further stratified in to MAP-positive and MAP-negative as determined by the detection of *IS*900 PCR in the DNA extracted from plaques. There was no significant

difference in the mean TVC value of these two groups (approx. 1 x 10^4 cfu ml^{-1}; p = 0.276) so the level of faecal contamination (high TVC) does not predict MAP status. Therefore the presence of MAP does not appear to correlate with the hygienic status of the milk, suggesting that faecal contamination is not the main route of entry of the MAP into the bulk milk. Similarly when TVC and the phage assay results are compared, there is no correlation (p = 0.270) between the number of plaques detected in a MAP-positive sample and the TVC (Fig. 2b) again indicating that a high plaque count, which associated with a high probability that MAP will be present in a sample (Botsaris et al., 2013), is not due to faecal contamination. These results are in agreement with a previous study that showed that there was no correlation between the total bacteriological count, somatic cell count and the detection of IS900 sequences by PCR in a survey of bulk tank milk (Corti and Stephan, 2002) and therefore faecal contamination alone cannot account for the presence of MAP in raw milk but suggests that these cells are being directly shed into the milk.

3.2.2 Evidence for asymmetric distribution of MAP in raw milk due to intracellular location

If MAP is not present in the milk due to faecal contamination, it must have entered the milk by a different route. As an intracellular pathogen, one such route is via infected somatic cells which are shed into the milk. Since it has been determined that MAP is not killed by pasteurisation as effectively in naturally infected milk compared to results gained using experimentally inoculated milk, the intracellular location of the MAP cells in naturally infected milk could be providing protection for the cells against the heat of treatment.

To provide evidence that the MAP being detected in raw milk were primarily intracellular, raw bulk tank milk was obtained from a farm with a known Johne's disease problem which was likely to contain viable MAP cells. One litre of raw milk was taken and split into twenty 50 ml samples and ten of these were tested using the phage assay for the presence of mycobacteria (termed 'whole' samples). The other ten samples were prepared for the phage assay in the same way, but prior to conducting the phage assay were washed with MP which lyses any somatic cell present and releases intracellular

mycobacteria (Swift et al., 2013). At this point each of these samples were further split into two 25 ml portions (termed 'paired' samples) and each was tested separately using the phage assay (Fig. 4A). Figure 4B shows that for each of the two groups of samples, the median number of mycobacteria detected was the same (50 vs 49) and also the range of the plaque numbers detected within each group was very similar (range for 'whole' samples = 16-145 per 50 ml; range for paired samples = 13-131 per 50 ml). These results showed that processing the samples in two different ways did not affect the overall number of mycobacteria detected.

When the numbers of mycobacteria per 50 ml replicate sample for the split samples were examined, it can be seen that there is a good correlation between the number of mycobacteria detected ($r^2 = 0.79$) for each of the pairs (Fig. 4C). However it is not possible to carry out a simple correlation for the replicate 'whole' samples as there is no non-arbitrary method that can be used to pair them. Hence two analyses were performed to examine a null hypothesis of complete spatial randomness (CSR) of cell distribution within the milk when samples are split into sub-samples by these two different methods. The first examined the number of bacteria present in each of the 50 ml samples, including the counts formed by summing the counts in each of the two paired samples, giving 20 samples in total. To analyse the initial allocation into 50 ml sub-samples, a Pearson Chi-squared test was used, in which there are 19 d.f. and the predicted count for each sample would be 59.4, which is the total count in the whole sample divided by 20. This test essentially assumes that the counts in each subsample are drawn from a Poisson distribution with mean equal to 59.4. The result of this analysis was that this null hypothesis of CSR across the subsamples was strongly rejected ($P<0.001$) and suggests that the distribution of mycobacteria present in the bulk tank milk is uneven so that when an individual 50 ml sample is taken there is likely to be a significant variation in the numbers of mycobacteria detected.

The second used a permutation test, to compare the total bacterial count in the paired samples with that expected from a random (binomial) allocation. Expressing each of the 10 allocations as a

proportion $p_i=N_{1i}/N_{Ti}$ of the total $N_{Ti}=N_{1i}+N_{2i}$ of the two halves for that sample, 10,000 sets of equivalent proportions were generated from a binomial distribution. Each of these sets takes the form $p_i^*=r_i/N_{Ti}$ where $r_i \sim B(N_{Ti},0.5)$ for i=1,...10, thus being a random allocation of the same total count as the corresponding sample. For each of these sets, a summary statistic - in this case the variance of these proportions- is calculated. The position (order statistic) of the variance of the proportions from the data within this list of 10,000 variances from the binomial allocations, gives the p-value. As a two-tailed test is to be used, the smaller or the two order statistics are taken and doubled to obtain the final p-value, which was p=0.625. Thus for the split samples the null hypothesis of randomness cannot be rejected, since the evidence of randomness seen in the initial analysis is no longer present after this lysis step has been carried out.

Since the only difference between the two treatments was the lysis of the somatic cells to release any intracellular mycobacteria before the paired samples were split (see Fig. 4A), and the overall plaque number detected was not affected by the change in sample preparation method, this pattern could be explained if the mycobacteria in the original milk were inside somatic cells rather being than free in the milk matrix, explaining the heterogeneous distribution of MAP cells in the 50 ml milk samples.

Taken with the results which indicate that the MAP cells are not introduced into the milk by faecal contamination, the results from these experiments suggest that the MAP cells in raw milk are primarily intracellular, located within infected somatic cells which have been shed into the milk, although further experiments are required to confirm this hypothesis. The results of the survey carried out in this study, and the results of other published surveys of retail pasteurised milk, clearly show that MAP in retail milk is able to survive pasteurisation. Laboratory studies of heat inactivation of MAP milk have consistently shown that HTST pasteurisation conditions are effective at reducing the levels of viable MAP by 3-6 $\log_{10}$ (McDonald et al., 2005). Since levels of MAP previously reported in milk from infected cows are generally not reported to be more than 10^2 cfu ml^{-1} MAP (Slana et al., 2008; Sweeney et al., 1992), pasteurisation should achieve complete inactivation of MAP. However these heat

inactivation studies use laboratory grown bacteria that were artificially added to milk. Our results suggest that MAP in raw milk is primarily located inside somatic cells which are made up of polymorphonuclear leukocytes, macrophages, lymphocytes and a small number of mammary epithelial cells (Boutinaud and Jammes, 2002). It has previously been shown that MAP surviving inside amoeba are approximately 2-fold more resistant to chorine disinfection (Whan et al., 2006) and this in part is due to the physical protection provided by the large mass of the host cell surrounding the bacteria (amoeba = 15-35 µm). Protection against chlorine disinfection has also been reported for a range of internalised bacteria, especially when they are located within vacuoles (King et al., 1988). It is known that, like many other intracellular pathogens, MAP can be found inside vacuoles of infected macrophage (Bannantine and Stabel, 2002). Since the somatic cells in milk generally range in size from 10-20 µm, and therefore are of a similar size to the amoebae used in the disinfection studies, it is possible that the survival of low levels of MAP during pasteurisation of naturally infected milk is due to their intracellular location, with the somatic cell providing sufficient protection to prevent complete inactivation of the bacteria. Patel et al. (2006) have shown that bovine mammary epithelial cells can contain up to 10^4 MAP cells after infection, and MAP are known to be able to replicate within macrophage (Arsenault et al., 2014), so it is also likely that infected somatic cells will not contain only a single MAP cell, increasing the likelihood that the low numbers of survivors could represent a residual population of survivors that have been physically protected from the heat treatment.

4. Conclusion

The association between MAP and Crohn's disease has been discussed for many years (Feller et al., 2007; Rhodes et al., 2014), with pasteurised milk thought to be a key vehicle of transmission to humans. One of the advantages of using phage to detect bacteria is that the bacterial cell must be viable before detection can occur and therefore, unlike PCR-based methods, differentiates between live and dead cells (Stanley et al., 2007; Swift et al., 2014). This makes the method very useful for

studying pasteurised products which may contain inactivated cells. In this study the phage-PCR assay was shown to be able to detect low levels of MAP in pasteurised milk with 10 % of the samples found to contain viable MAP. The prevalence of MAP was higher than that reported in a number of other published studies that used culture to detect MAP (1.7 to 6.7%) but this does not suggest that our samples had a higher prevalence rate, just that this method is more sensitive and therefore increases the frequency with which samples containing very low numbers of cells give a positive result. Given the rapidity of this method, there is now potential for the food industry to use this method to monitor the efficacy of pasteurisation processes. There is a clear need for further research to be carried out to fully understand this mechanism of survival and the fact that mycobacteria that reside inside eukaryotic cells can be protected from external sources of stress. Here we have demonstrated the ability to rapidly detect and enumerate MAP using the phage assay providing a useful tool to allow such studies to be completed without the need for prolonged incubation of cultures required when using traditional culture methods.

Acknowledgements

Z.E.G was funded by a joint studentship between the University of Nottingham and SRUC. B.M.C.S was funding by a University of Nottingham Hermes Innovation. G.B was sponsored by an EU research scholarship. Thanks is extended to staff and students who supplied samples of retail pasteurised milk samples for the study, and to a local farm for supplying the bulk tank milk. SRUC receives support from the Scottish Government

References

Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Dennis, M.M., Gould, D.H., Salman, M.D., 2008. Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection

in dairy cattle and its association with antemortem test results. *Veterinary Microbiology* 127, 300-308.

Arsenault, R.J., Maattanen, P., Daigle, J., Potter, A., Griebel, P., Napper, S., 2014. From mouth to macrophage: mechanisms of innate immune subversion by *Mycobacterium avium* subsp *paratuberculosis*. *Veterinary Research* 45.

Ayele, W.Y., Svastova, P., Roubal, P., Bartos, M., Pavlik, I., 2005. *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic. *Applied and Environmental Microbiology*. 71, 1210-1214.

Bannantine, J.P., Stabel, J.R., 2002. Killing of *Mycobacterium avium* subspecies *paratuberculosis* within macrophages. *BMC Microbiology* 2.

Botsaris, G., Liapi, M., Kakogiannis, C., Dodd, C.E.R., Rees, C.E.D., 2013. Detection of *Mycobacterium avium* subsp *paratuberculosis* in bulk tank milk by combined phage-PCR assay: Evidence that plaque number is a good predictor of MAP. I*nternational Journal of Food Microbiology*. 164, 76-80.

Botsaris, G., Slana, I., Liapi, M., Dodd, C., Economides, C., Rees, C., Pavlik, I., 2010. Rapid detection methods for viable *Mycobacterium avium* subspecies *paratuberculosis* in milk and cheese. *International Journal of Food Microbiology*. 141, S87-S90.

Botsaris, G., Swift, B.M., Slana, I., Liapi, M., Christodoulou, M., Hatzitofi, M., Christodoulou, V., Rees, C.E., 2016. Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in powdered infant formula by phage-PCR and confirmed by culture. *Internatinoal Journal of Food Microbiology*. 216, 91-94.

Boutinaud, M., Jammes, H., 2002. Potential uses of milk epithelial cells: a review. *Reproduction Nutrition Development*. 42, 133-147.

Bradner, L., Robbe-Austerman, S., Beitz, D.C., Stabel, J.R., 2013. Optimization of Hexadecylpyridinium Chloride Decontamination for Culture of *Mycobacterium avium s*ubsp. *paratuberculosis* from Milk. *Journal of Clinical Microbiology*. 51, 2804-2804.

Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R., Hermon-Taylor, J., 2003. Detection and verification of *Mycobacterium avium* subsp *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *Journal of Clinical Microbiology*. 41, 2915-2923.

Chiodini, R.J., Hermontaylor, J., 1993. The Thermal-Resistance of *Mycobacterium-Paratuberculosis* in Raw-Milk under Conditions Simulating Pasteurization. *Journal of Veterinary Diagnostic Investigation*. 5, 629-631.

Corti, S., Stephan, R., 2002. Detection of *Mycobacterium avium* subspecies *paratuberculosis* specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. *BMC Microbiology* 2.

Dundee, L., Grant, I.R., Ball, H.J., Rowe, M.T., 2001. Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp *paratuberculosis* from milk. *Letters in Applied Microbiology*. 33, 173-177.

Feller, M., Huwiler, K., Stephan, R., Altpeter, E., Shang, A., Furrer, H., Pfyffer, G.E., Jemmi, T., Baumgartner, A., Egger, M., 2007. *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. *Lancet Infectious Diseases*. 7, 607-613.

Grant, I.R., Ball, H.J., Neill, S.D., Rowe, M.T., 1996. Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures. *Applied and Environmental Microbiology*. 62, 631-636.

Grant, I.R., Ball, H.J., Rowe, M.T., 2002a. Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. *Applied and Environmental Microbiology*. 68, 2428-2435.

Grant, I.R., Hitchings, E.I., McCartney, A., Ferguson, F., Rowe, M.T., 2002b. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. *Applied and Environmental Microbiology*. 68, 602-607.

King, C.H., Shotts, E.B., Wooley, R.E., Porter, K.G., 1988. Survival of Coliforms and Bacterial Pathogens within Protozoa during Chlorination. *Applied and Environmental Microbiology*. 54, 3023-3033.

Logar, K., Kopinc, R., Bandelj, P., Staric, J., Lapanje, A., Ocepek, M., 2012. Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of *Mycobacterium avium* subsp *paratuberculosis* in subclinically infected dairy cattle: comparison with faecal culture, milk real-time PCR and milk ELISA. *BMC Veterinary Research*. 8.

McDonald, W.L., O'Riley, K.J., Schroen, C.J., Condron, R.J., 2005. Heat inactivation of *Mycobacterium avium* subsp *paratuberculosis* in milk. *Applied and Environmental Microbiology*. 71, 1785-1789.

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T., HermonTaylor, J., 1996. IS*900* PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cows' milk in England and Wales. *Applied and Environmental Microbiology*. 62, 3446-3452.

Mole, R., Trollip, A., Abrahams, C., Bosman, M., Albert, H., 2007. Improved contamination control for a rapid phage-based rifampicin resistance test for *Mycobacterium tuberculosis. Journal of Medical Microbiology*. 56, 1334-1339.

NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS (2010) Assessment of Food as a Source of Exposure to Mycobacterium avium subspecies paratuberculosis (MAP) *Journal of Food Protecti*on, 73, 1357–1397

Paolicchi, F., Cirone, K., Morsella, C., Gioffre, A., 2012. First Isolation of *Mycobacterium Avium* Subsp *Paratuberculosis* from Commercial Pasteurized Milk in Argentina. *Brazilian Journal of Microbiology*. 43, 1034-1037.

Patel, D., Danelishvili, L., Yamazaki, Y., Alonso, M., Paustian, M.L., Bannantine, J.P., Meunier-Goddik, L., Bermudez, L.E., 2006. The ability of *Mycobacterium avium* subsp *paratuberculosis* to enter bovine epithelial cells is influenced by preexposure to a hyperosmolar environment and intracellular passage in bovine mammary epithelial cells. *Infection and Immunity*. 74, 2849-2855.

Rademaker, J.L.W., Vissers, M.M.M., Giffel, M.C.T., 2007. Effective heat inactivation of *Mycobacterium avium* subsp *paratuberculosis* in raw milk contaminated with naturally infected feces. *Applied and Environmental Microbiology*. 73, 4185-4190.

Rhodes, G., Richardson, H., Hermon-Taylor, J., Weightman, A., Higham, A., Pickup, R., 2014. *Mycobacterium avium* Subspecies *paratuberculosis*: Human Exposure through Environmental and Domestic Aerosols. *Pathogens*. 3, 557-595.

Slana, I., Kralik, P., Kralova, A., Pavlik, I., 2008. On-farm spread of *Mycobacterium avium* subsp *paratuberculosis* in raw milk studied by IS*900* and F57 competitive real time quantitative PCR and culture examination. *International Journal of Food Microbiology*. 128, 250-257.

Stabel, J.R., Lambertz, A., 2004. Efficacy of pasteurization conditions for the inactivation of *Mycobacterium* avium *subsp paratuberculosis* in milk. *Journal of Food Protection*. 67, 2719-2726.

Stanley, E.C., Mole, R.J., Smith, R.J., Glenn, S.M., Barer, M.R., McGowan, M., Rees, C.E.D., 2007. Development of a new, combined rapid method using phage and PCR for detection and identification of viable *Mycobacterium paratuberculosis* bacteria within 48 hours. *Applied and Environmental Microbiology*. 73, 1851-1857.

Sweeney, R.W., Collins, M.T., Koets, A.P., McGuirk, S.M., Roussel, A.J., 2012. Paratuberculosis (Johne's Disease) in Cattle and Other Susceptible Species. *Journal of Veterinary Internal Medicine*. 26, 1239-1250.

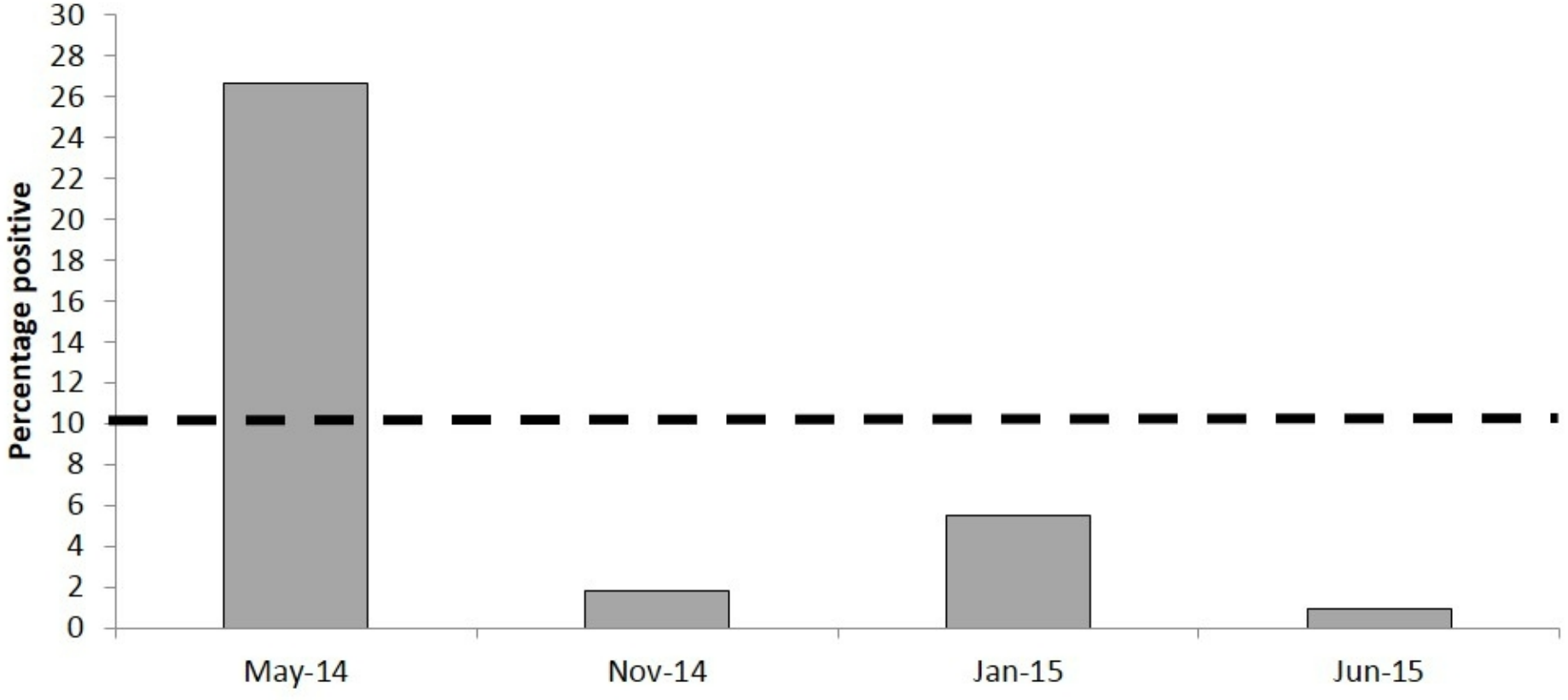
Sweeney, R.W., Whitlock, R.H., Rosenberger, A.E., 1992. Mycobacterium-Paratuberculosis Cultured from Milk and Supramammary Lymph-Nodes of Infected Asymptomatic Cows. *Journal of Clinical Microbiology*. 30, 166-171.

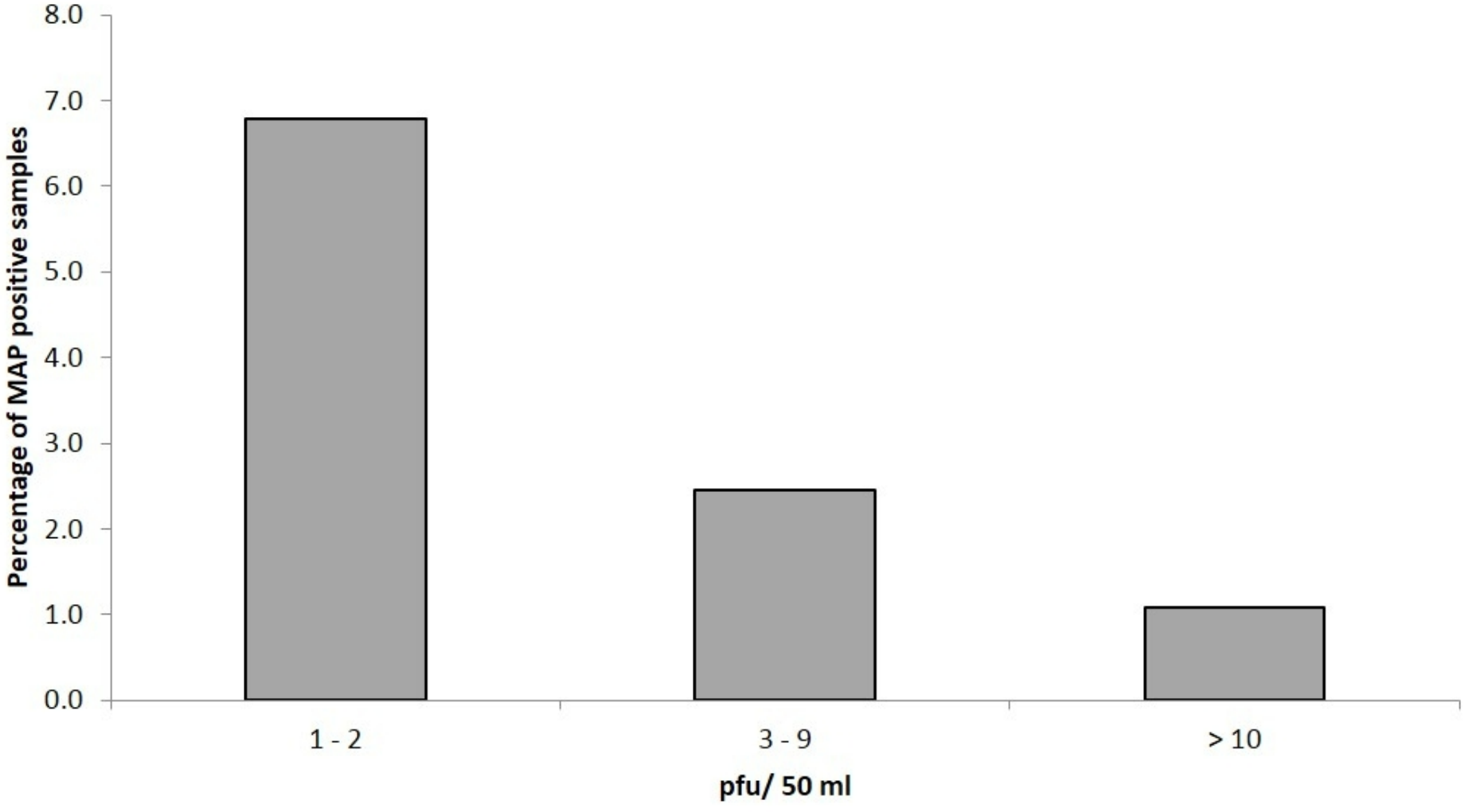
Swift, B.M., Denton, E.J., Mahendran, S.A., Huxley, J.N., Rees, C.E., 2013. Development of a rapid phage-based method for the detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in blood within 48 h. *Journal of Microbiological Methods*. 94, 175-179.

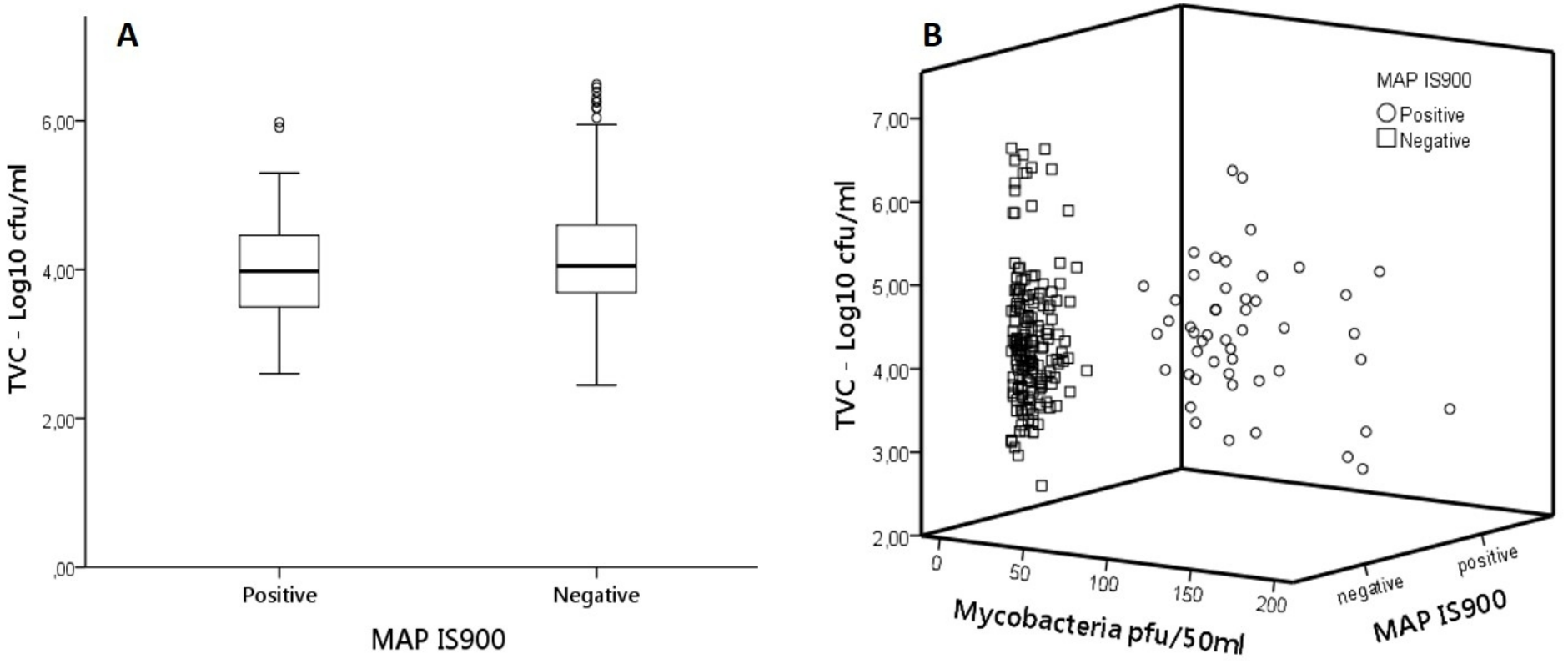
Swift, B.M., Gerrard, Z.E., Huxley, J.N., Rees, C.E., 2014. Factors Affecting Phage D29 Infection: A Tool to Investigate Different Growth States of Mycobacteria. *PLoS One*. 9, e106690.

Swift, B.M., Rees, C.E., 2013. Detecting mycobacteria in cattle blood. *Veterinary Record*. 173, 522-523.

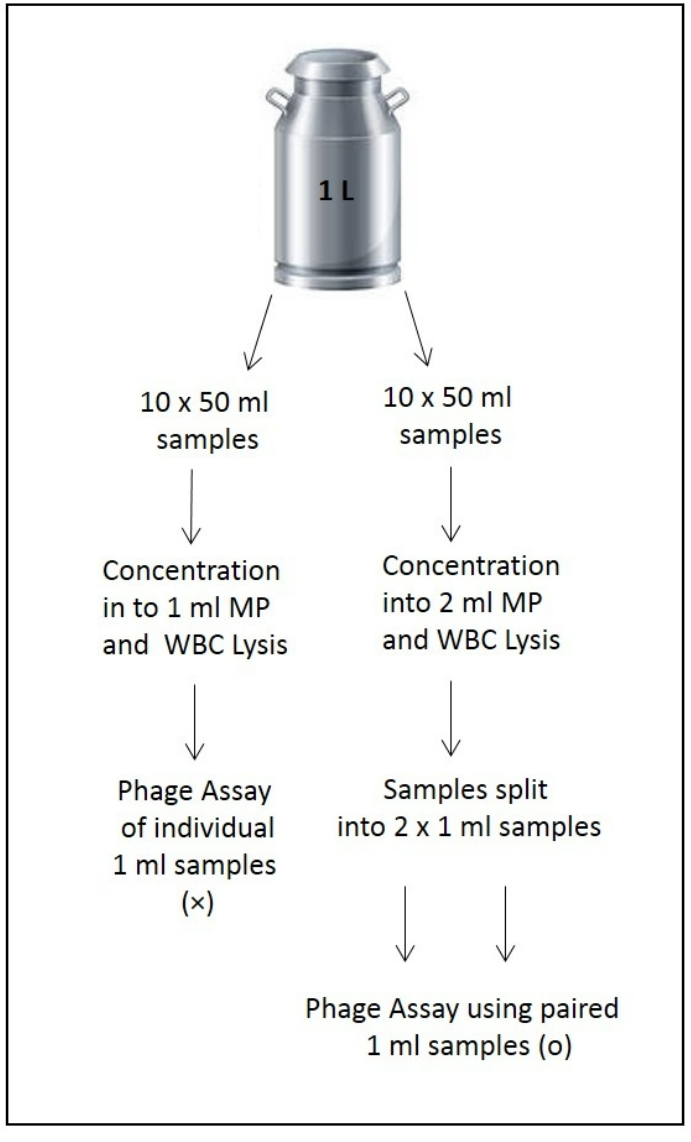
Whan, L., Grant, I.R., Rowe, M.T., 2006. Interaction between *Mycobacterium avium* subsp *paratuberculosis* and environmental protozoa. *BMCMicrobiology*. 6.


Percentage positive
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
May-14
Nov-14
Jan-15
Jun-15

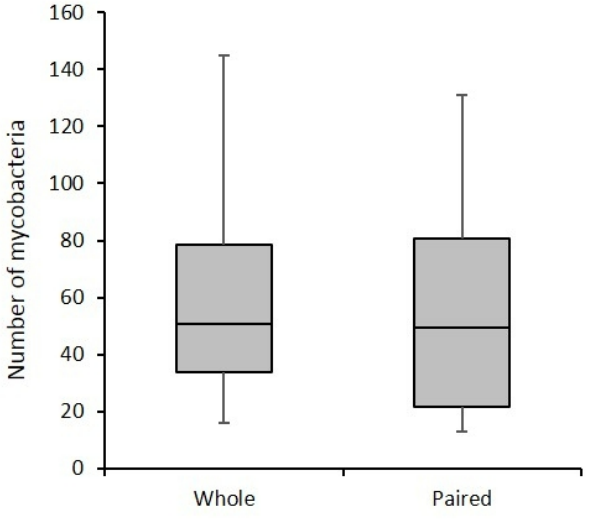

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Percentage of MAP positive samples
1 - 2
3 - 9
> 10
pfu/ 50 ml


A
6,00
4,00
2,00
,00
TVC - Log10 cfu/ml
Positive
Negative
MAP IS900
B
7,00
6,00
5,00
4,00
3,00
2,00
TVC - Log10 cfu/ml
MAP IS900
Positive
Negative
0
50
100
150
200
Mycobacteria pfu/50ml
negative
positive
MAP IS900

A


1 L
10 x 50 ml samples
10 x 50 ml samples
Concentration in to 1 ml MP and WBC Lysis
Concentration into 2 ml MP and WBC Lysis
Phage Assay of individual 1 ml samples (×)
Samples split into 2 x 1 ml samples
Phage Assay using paired 1 ml samples (o)

B


Number of mycobacteria
160
140
120
100
80
60
40
20
0
Whole
Paired

C

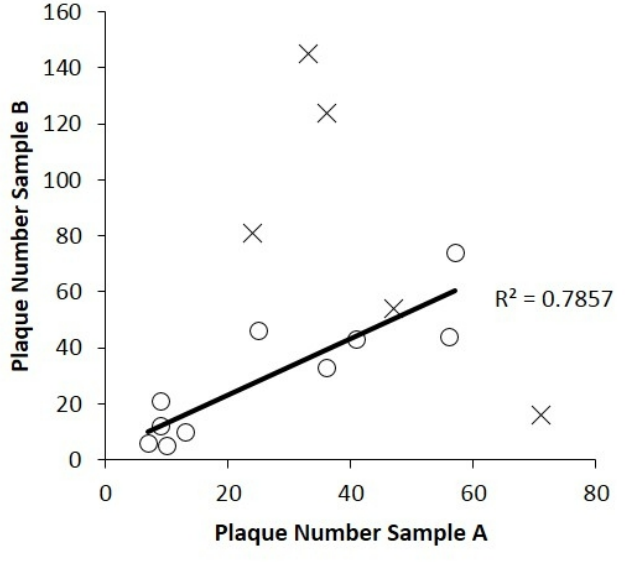

Plaque Number Sample B
160
140
120
100
80
60
40
20
0
0
20
40
60
80
Plaque Number Sample A
R² = 0.7857

Figure 1. Prevalence of viable MAP in retail pasteurised semi-skinned milk

Graph showing the percentage of MAP-positive (phage-PCR positive) samples detected at each sampling point during the survey (total number of samples = 368). Samples of retail semi-skimmed milk (1.7 % fat) samples were provided by volunteers primarily from three UK counties (Nottinghamshire, Derbyshire, and Leicestershire). The dashed line indicates the overall percentage of MAP-positive samples detected in the survey (10.3 %).

Figure 2. Distribution of the number of viable MAP samples across the whole survey

A total of 38 MAP-positive samples were detected, defined as those that produced plaques and were IS900-PCR positive. Data was grouped by plaque number per 50 ml sample and the results for each group reported as percentage of the total sample set (n = 368 semi skimmed (1.7 % fat) milk). The groupings were based on the reported sensitivities of other methods *i.e.* 1-2 cells; no other method reports being able to detect this low level, 3-9 cells and >10 cells; potentially detectable by direct PCR; >10 cells; potentially detectable by culture.

Figure 3. Comparison of *MAP* status and TVC of bulk milk samples

Samples of raw bulk tank milk (n = 225) were tested using the phage-PCR assay and the TVC of each sample was separately determined. Samples were classified as MAP-positive or MAP-negative according to the results of the Phage-IS*900* PCR assay. Panel A. For each data set, the distribution of TVC values is represented by a Box and Whisker plot. An independent samples t-test revealed no significant difference between the TVC results of the MAP-positive samples and the MAP-negative samples (p=0.276). Panel B. MAP-positive samples are shown as open circles and MAP-negative samples are shown as open squares. For each group, the distribution of TVC values is compared with the plaque count for individual samples. A Pearson correlation indicated no significant relationship existed between these two variables (p=0.270).

Figure 4. Distribution of viable MAP in whole milk samples with and without somatic cell lysis

Panel A. Summary diagram of the experimental design. One litre of raw milk split into twenty 50 ml samples. Ten of these were centrifuged and the pellet resuspended in 1 ml MP to lyse the somatic cells and then tested using the phage assay for the presence of mycobacteria ('whole' samples). The other ten samples were centrifuged, and the pellet resuspended in 2 ml MP to lyse the somatic cells. This sample was further split into two 1 ml samples ('paired' samples) before each one was tested separately using the phage assay. Panel B. Box and Whisker plot showing the median number and range of mycobacteria detected for 'whole' (mean 50, range 16 to 145) and 'paired' (mean 49, range 13 to 131) samples for the two different treatments. To normalise the data the results for the paired samples were multiplied by 2 to determine the median and range of data. Panel C. Plot to show the correlation between plaque numbers detected in 'paired' samples ($r^2 = 0.79$)