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# Clinical presentation and diagnostics of cattle with suspected Johne's disease

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# Abstract

Johne's disease, caused by the bacteria *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic, wasting condition of ruminants, which is characterized by the clinical signs of weight loss and diarrhoea. The economic losses associated with the disease in the cattle and sheep industry, worldwide spread and high prevalence make Johne's disease an important disease to investigate. Additionally, MAP has been isolated from humans suffering of Crohn's disease and zoonotic potential of MAP in humans is still under debate. Currently available diagnostic tests have impaired sensitivity, particularly in preclinical stages of disease and thus a prompt and effective identification of infected animals is difficult. The primary aim of this work was to analyse and describe the history, clinical presentation and diagnostic test results of cattle submitted to a veterinary teaching hospital that showed clinical signs consistent with Johne's disease. The secondary aim was to compare diagnostic test results to the gold standard, faecal culture.

Fifty-nine out of the 192 cattle referred over 18 months met the inclusion criteria for the study, which was poor body condition or a history of weight loss and diarrhoea. A total of 52 females and seven males, comprising 34 beef and 25 dairy cattle were studied. All cattle had a full clinical examination, biochemistry blood analysis, Johne's blood serology and faecal PCR and culture. They were inspected post-mortem and the ileo-caecal valve and proximal lymph node were examined histopathologically using a haematoxylin-eosin (H/E) and Ziehl-Neelsen (ZN) staining. Results from clinical examination, albumin and globulin serum concentrations, Johne's serology, PCR, post-mortem examination and histopathology examination were compared to faecal culture results. Thus, this study reported all the clinical and diagnostic test results commercially available for Johne's disease in cattle, such study was not reported before. Results showed inconsistent results between PCR and culture tests. However, both blood serology and H/E slide examination tests had a specificity of 100%.

This work should help inform clinicians on interpretation of Johne's diagnostic testing in clinical cases and may have implications for Johne's control programmes relying on the results from the diagnostic tests reported.

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# Acknowledgement

To the farm animal residents, Richard Vazquez, Francisco Malcata, Andrea Francesio and Nicola Gladden, which I have shared the office with and have helped me with new ideas, sharing their projects, skills, literature and experience. Thanks for making these two years easier and helping in the downs.

Finally, thanks to my family, which have supported me throughout these two years of work and have encouraged me to keep working calmly in the difficult moments.

# Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Joan Baró Basora

# Abbreviations

| | |
|---|---|
| **BCS** | Body condition score |
| **BTB** | Bovine Tuberculosis |
| **BVMS** | Bachelor of Veterinary Medicine and Surgery |
| **CD** | Crohn's disease |
| **CFU** | Colony-forming unit |
| **CHeCS** | Cattle Health Certification Standards |
| **CI** | Confidence interval |
| **CT** | Computed tomography |
| **CT value** | Cycle-threshold, in relation to the PCR test |
| **DC** | Dendritic cell |
| **DEFRA** | Department for Environment Food and Rural Affairs |
| **DIM** | Days in milk |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **FSAI** | Food Safety Authority of Ireland |
| **GSHPx** | Glutathione peroxidase |
| **H/E** | Haematoxylin-eosin |

**HEYM**        Herrold's egg yolk medium

**IDTT**         Intradermal tuberculin test

**IFN-g**        Interferon gamma

**IgG1**         Immunoglobulin G1

**MAP**         *Mycobacterium avium* subsp. *paratuberculosis*

**MID**         Minimum infectious dose

**MRI**         Magnetic resonance imaging

**NMR**        National Milk Records

**NPV**         Negative predictive value

**NSAID**       Nonsteroidal Anti-inflammatory Drug

**OIE**         World Organisation for Animal Health. Originally from the French, "Office international des epizooties".

**OR**          Odds Ratio

**PCR**         Polymerase-chain reaction

**PCV**         Packed cell volume

**PM**          Post-mortem

**PME**         Post-mortem examination

**PPD**         Purified protein derivate

**PPV**         Positive predictive value

| | |
|---|---|
| **qPCR** | Quantitative PCR |
| **Se** | Sensitivity |
| **SNP** | Single Nucleotide Polymorphism |
| **Sp** | Specificity |
| **SRUC** | Scotland's Rural College |
| **SVM** | School of Veterinary Medicine |
| **Th1** | T-helper type 1 (cell/immune response) |
| **Th2** | T-helper type 2 (cell/immune response) |
| **TP** | Total protein |
| **UK** | United Kingdom |
| **USA** | United States of America |
| **ZN** | Ziehl-Neelsen |

# Chapter 1     Introduction and Literature Review

## 1.1 History and context

### 1.1.1 First time described

The first description of Johne's disease in cattle was made in 1826 . It was described as an intestinal disorder that caused a chronic and debilitating disease in cows (Chiodini, 2005). It was not until 1895, when Dr. Johne and Dr. Frothingham, from Dresden (Germany) when the disease was reported again as a peculiar case of tuberculosis (Clarke, 1997). They performed a post-mortem examination on a cow with a history of weight loss and diarrhoea and noticed the thickened intestinal mucosa and enlarged mesenteric lymph nodes (typical necropsy lesions of Johne's disease). Histological examination and acid-fast staining showed acid-fast positive bacteria. Although a sample of infected tissue failed to cause tuberculosis when injected to guinea pigs, Johne and Frothingham concluded that the disease observed in the cow was caused by the bacterium that causes tuberculosis in birds (*Mycobacterium avium*) and named the condition "pseudotuberculous enteritis" (Chiodini, 2005).

In 1906, Bang realised that animals showing "pseudotuberculosis" tested negative to the intradermal bovine tuberculin test but positive to avian tuberculin (Chiodini, 2005). The organism causing avian tuberculosis could be grown on laboratory culture from suspected cases but the specific organism causing pseudotuberculosis could not. Bang was the first one to refer to this disease as Johne's disease (Clarke, 1997).

The British scientist F.W. Twort made many efforts in order to isolate the aetiological agent of Johne's disease (Chiodini, 2005). He noted small bacterial colonies growing like satellites around larger colonies in old cultures of *Mycobacterium phlei* he was preparing to discard. Twort incorporated a heat-killed preparation of *M. phlei* into his culture medium, which supported the growth of a new acid-fast bacterium in 1910 (Clarke, 1997). Twort then successfully fulfilled Koch's postulates by growing *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) in the laboratory and reproducing the disease in experimentally infected cattle (Harris and Barletta, 2001).

The organism, which was originally named *Mycobacterium enteritidis chronicae pseudotuberculosae bovis johne* in 1910, it was renamed as *Mycobacterium paratuberculosis* in 1923 and then *Mycobacterium johnei* in 1951, which coexisted with the previous name. In 1990, due to its phylogeny, it took the name of *Mycobacterium avium* subspecies *paratuberculosis* in which is the name that is currently used (Harris and Barletta, 2001).

## 1.2 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) – the bacteria

### 1.2.1 Description of the bacteria

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a slow-growing, rod-shaped, gram-positive, acid-fast, non-spore forming and non-motile organism belonging to the species *M. avium*. (Harris and Barletta, 2001). They are obligate aerobes, which usually require the presence of mycobactin J, an iron-chelating agent, for growth *in vitro*. However, some other *M. avium* strains are also mycobactin-dependent. Visible growth usually takes 8–12 weeks at 37°C (Nielsen, 2009).

The mycobacterial envelope consists of four layers (Tessema *et al.*, 2001). They are listed below from innermost to outermost (Figure 1-1):

- The plasma membrane, like that of other bacteria, is composed of a lipid bilayer with embedded membrane proteins (Tessema *et al.*, 2001).

- Electron-dense layer, which appears dense on transmission electron microscopy, consists of a peptidoglycan backbone covalently linked through a diglycosylphosphoryl bridge to a branched chain of arabinogalactan (Tessema *et al.*, 2001).

- Electron-transparent layer is mainly composed of mycolic acids that are esterified to the arabinogalactan (Tessema *et al.*, 2001).

- Outer layer: mainly formed by glycolipids such as Lipoarabinomannan, which is a highly immunogenic and potent inhibitor of macrophage activation that down-regulates macrophage effector function at several levels (Tessema *et al.*, 2001).
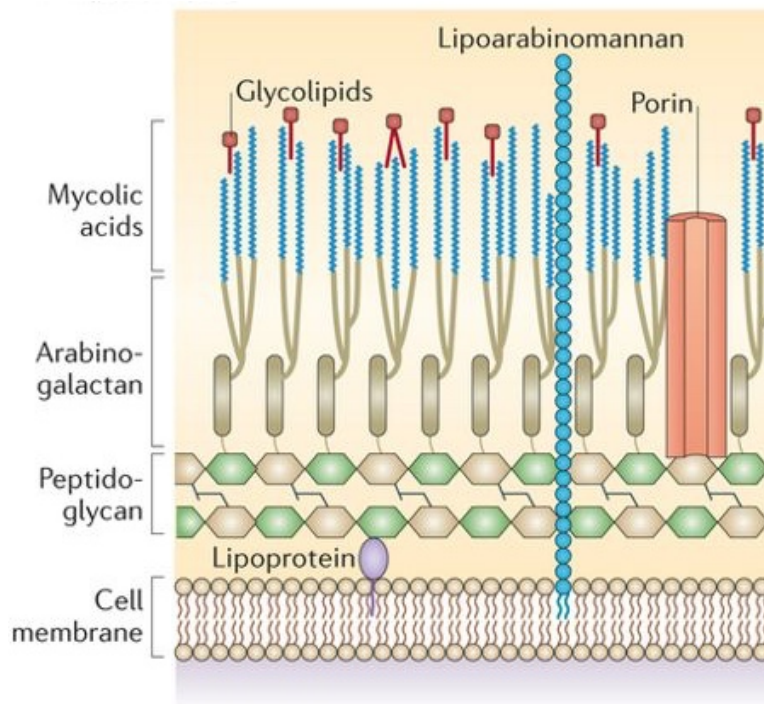
**Figure 1–1 Cell wall structure of mycobacteria. (Adapted from Brown *et al.*, 2015)**

## 1.2.2 Mycobacterium phylogenesis

MAP are a mycobacteria classified in the slow growing mycobacteria phylogenetic group (Figure 1-2). Other well-known pathogens in the same group are *M. tuberculosis* and *M. bovis*, causative agents of tuberculosis in humans and cattle respectively. MAP are also related to *Mycobacterium leprae*, the cause of leprosy in humans. MAP share certain biological characteristics in common with these mycobacterial pathogens such as the insidious course of the disease, a long incubation period, limited effective immune response from the host and the formation of granulomatous inflammatory response.
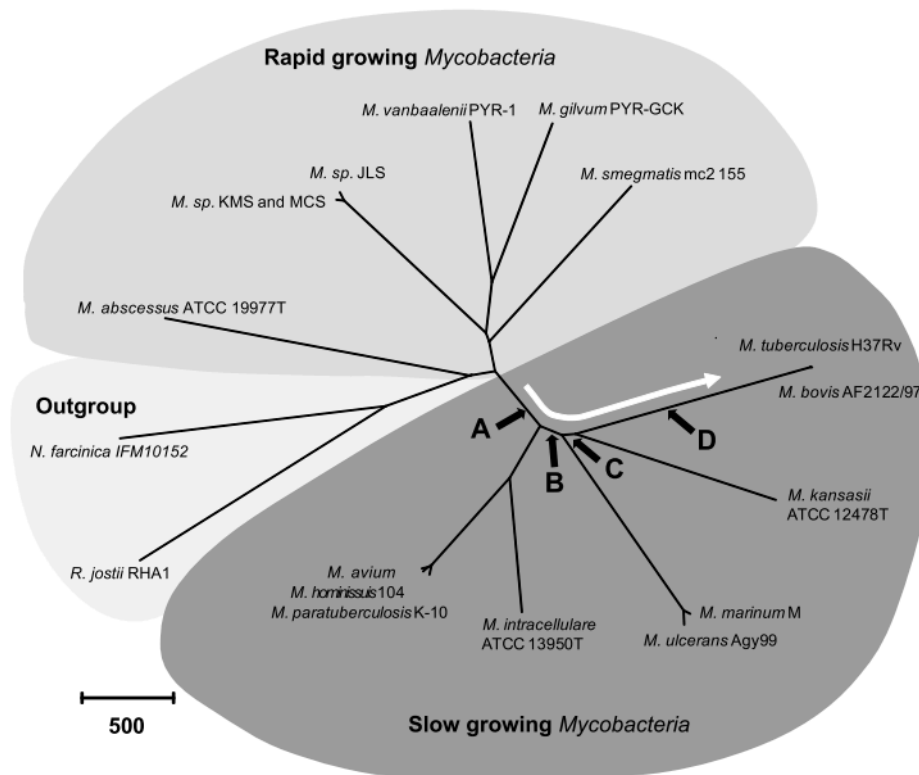
**Figure 1–2** *Mycobacteria* **spp. phylogenetic relationship (Veyrier** *et al.***, 2009)**

## 1.2.3 MAP genotypes: types of MAP and virulence

Attempts have been made to classify MAP strains. The first classification was performed in relation to the species where each strain was isolated from. This classification system is still used. The different strains are:

- Type S: first isolated from sheep. They have been isolated from cattle too, but in much lesser extent than in sheep, which suggests that cattle might need a higher Type S burden to develop infection (Bryant *et al.*, 2016).

- Type C: first isolated from cattle, but it has been found in a large number of different hosts, which seems to indicate that they do not have a host preference (Bryant *et al.*, 2016).

- Type B:  first isolated from buffalos. Recently it has been demonstrated that they are a subtype of "Type C" strain and they are not restricted to *Bison* species (Bryant *et al.*, 2016).

Moloney and Whittington (2008), after a study in Australia with sheep and cattle co-grazing, concluded that Type S infection in cattle is possible but sporadic in nature. Low prevalence of Type S strain in cattle suggests that it is unlikely that this strain could persist in a cattle herd without ongoing exposure from infected sheep.

Genotyping of the MAP strains allowed classification of different strains in relation to genetic similarities and another classification was proposed (Stevenson, 2010).This classification system can be used on its own or complementing the system described before (Figure 1-3).

- Type I: is part of the "Type S" pigmented ovine strain, isolated from the UK by (Stevenson, 2015). Isolated only from sheep.

- Type II: from the "Type C" group. It has been isolated from different species, including humans (Bryant *et al.*, 2016).

- Type III: from the "Type S" group. It has been isolated from sheep, goats and deer.
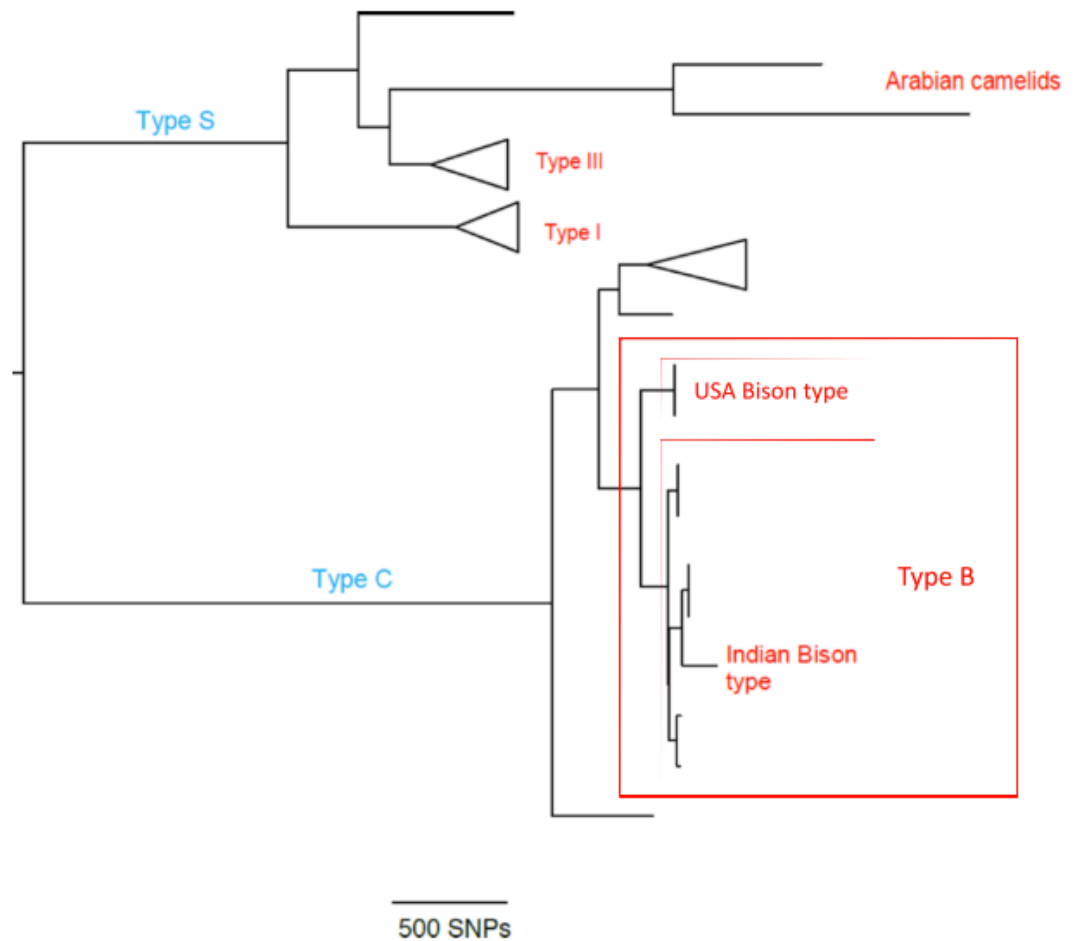
**Figure 1–3 Phylogenic tree of MAP strain types (Stevenson, 2015)**

Differences in virulence and mixed infections with more than one strain have not yet been completely understood (Barkema *et al.*, 2018). Different strains have been found to cause different lesions in lambs and mouse models, but there is still a lack of evidence of a strain-effect on the pathogenicity of MAP in cattle (Stevenson, 2015). This is a subject of further study that would allow veterinarians to better understand the pathogenicity of different strains.

## 1.2.4 Environment persistence

MAP can be found on pasture and it is excreted and carried by domestic and wild animals (Whittington *et al.*, 2004). MAP has been cultured from various locations within infected farms and it was even found in dust using electrostatic collectors at two meters from the floor by Eisenberg *et al.* (2015). It is known that MAP can survive in the environment for at least one year (Whittington *et al.*, 2004). Other studies have highlighted the survival of Type II MAP inoculated into sterile freshwater lake water columns, finding cultivable MAP even after 632 days

(Elliott *et al.*, 2015). Persistence of MAP in the environment is key to understanding MAP transmission in the herd and when implementing control measures at a herd level.

Some of the environmental factors that influence survival of MAP that have been studied are:

- Soil pH: application of lime to pasture (to increase soil pH) was shown to be protective against Johne's disease and associated with a 72% reduction in the number of positive cattle (Elliott *et al.*, 2015).

- Iron presence: showing a 1.4% increase in the risk of a herd being positive for every 1 parts per million (ppm) increase in soil iron content, and a 4% increase in the numbers of MAP-positive cows associated with every 10 ppm increase in soil iron content. Iron and pH are correlated- the higher the pH is the less iron is available (Elliott *et al.*, 2015).

- Temperature: temperatures below 0ºC, time below freezing point and oscillations in temperature impair MAP survival. On the other hand, the organism is resistant to heat and has been shown to survive high-temperature / short-time pasteurization (72°C for 15 seconds) (Grant *et al.*, 2002). Although batch pasteurization of colostrum at 60°C for 60 minutes is sufficient to eliminate MAP under most conditions (Godden *et al.*, 2006).

- Sunlight: Type I/III MAP strain persisted for a maximum of 55 weeks on soils and grasses when covered, but less than two weeks when fully exposed to sunlight in the absence of even plant cover (Whittington *et al.*, 2004).

A study carried out by Fecteau *et al.* (2013) investigated the persistence of MAP on farm. Manure samples were positive on culture samples on seven out of ten farms. After slurry spread was performed, soil, crop and feed samples were taken and they all were culture negative. However, they were all positive on PCR. Conclusions of the study highlighted that, following the spread of manure onto fields, MAP was found in soil, but less frequently in crops and silage samples and all the positive samples failed to grow MAP on culture.

When faced with the decision about where best to spread manure, fields used for production of crops intended for harvest are preferred to grazing pastures. In general, it appears that spreading of manure on fields prior to emergence of crops represents a low risk practice for the spread of infection on the farm (Fecteau *et al.*, 2013).

# 1.3 Crohn's disease

Crohn's disease (CD) is one of the major forms of inflammatory bowel disease in humans. It causes a chronic condition that is characterized by abdominal pain, weight loss and haematochezia. Incidence estimates suggest a range of 3.1-20.2 cases per 100,000 people per year and a prevalence of 201 cases per 100,000 population (Shaker, 2015). This condition is thought to be a heterogeneous and multifactorial disorder where genetic and immunological components are thought to be involved. Other risk factors such as smoking, high carbohydrate diet and nonsteroidal anti-inflammatory drugs (NSAIDs) intake have been proven (Shaker, 2015). The role of MAP in CD is still being discussed.

## 1.3.1 Link between MAP and Crohn's disease

The potential association between exposure to *Mycobacteria* spp. and inflammatory chronic disease, like CD in humans was first described by Burnham and Jones (1978). Later on Chiodini *et al*. (1984) isolated and described the Mycobacteria found in patients suffering from CD and inflammatory bowel disease. This bacteria was identified as MAP.

Further research on the subject has tried to prove a causal relationship between MAP and CD. Isolation of MAP from intestinal tissue of CD patients was achieved by Mishina *et al*. (1996). Naser *et al*. (2004) described a strong correlation between positive MAP (cultured from blood) and people suffering from CD. Although MAP was also found by PCR from blood of the control group, all culture results on the control group were negative. A recent study (Pierce, 2018) detected MAP by histopathology in intestines of people with CD, ulcerative colitis and different forms of colorectal cancer.

A study carried out in India (Chaubey *et al*., 2017) found that an unexpectedly high percentage of the population has had contact with MAP. Results from humans for blood PCR, stool PCR and MAP serology were 8.8%, 22.4 and 33.7% respectively. Moreover, MAP isolates from humans were "Indian Bison Type" strains, which is the most prevalent strain of MAP found in domestic ruminants in India. Other genetic studies found results that point towards the same direction. Bryant *et al*. (2016) found strong similarities between human-MAP isolates and

cattle-MAP isolates in the Netherlands and USA. In both cases, strains found in humans were very similar to the strains found in cattle in their respective countries. In this case the strains were "Type II".

Hill causative criteria have also been assessed in order to relate MAP as a causative or contributive agent of Crohn's disease. Hill's critera are: (i) strength of association ; (ii) consistency of effect ; (iii) specificity of effect ; (iv) temporality ; (v) biological gradient or dose response; (vi) biological plausibility. In a review of the subject performed by Uzoigwe et al. (2007) it was stated that the current epidemiological evidence strongly supports the conjecture that Crohn's disease is caused by MAP. Existing data showed that the MAP Crohn's disease phenomenon has fulfilled at least four (strength of association, consistency of effect, temporality and biological plausibility) of the six epidemiological causal criteria outlined by Hill (Uzoigwe et al. 2007).

On the other hand, some researchers state that evidence is not strong enough and Koch's postulates (isolation of the agent, disease replication in a healthy individual and re-isolation) have not been demonstrated. As a result, some people suggest that more evidence is needed before establishing a cause-effect relationship. Due to the multifactorial nature of CD and the ethical issues surrounding a human trial involving MAP inoculation of humans, this evidence will be difficult to find (Sweeney *et al.*, 2012; Chaubey *et al.*, 2017).

Considering all these results, it seems that MAP isolated in humans has the potential to come from MAP infected ruminants. Thus, measures to control human exposure to MAP should be enhanced until a definitive link is proven.

## 1.3.2 Diagnosis of Crohn's disease

Diagnosis of CD is difficult. The absence of a gold standard mean that different tests are normally needed to confirm CD. The diagnosis is confirmed by a clinical evaluation, history of the patient and a combination of:

- Endoscopy examination, looking for ulcerative lesions or other alterations of the mucosa(Van Assche *et al.*, 2010).

- Histopathology performed on a colon or ileum biopsy, looking for signs of granulomatous enteritis (Van Assche *et al.*, 2010).

- Magnetic resonance imaging (MRI), looking for thickening of the intestinal wall or inflammation (Van Assche *et al.*, 2010).

- Computed tomography (CT), looking for thickening of the intestinal wall (Van Assche *et al.*, 2010).

- Compatible biochemical results as faecal calprotectin, which identifies intestinal inflammation(Van Assche *et al.*, 2010).

Hence, a comprehensive and holistic approach is needed in order to diagnose every patient (Van Assche *et al.*, 2010; Shaker, 2015). A similar approach, but with other diagnostic methods is used in cattle with Johne's disease, where due to a lack of sensitivity of the available tests, a combination of factors (history, clinical exam and test results) are taken into account to diagnose Johne's disease.

## 1.3.3 Human exposure to MAP

The confirmed presence of viable MAP in pasteurized milk for human consumption in different countries has led to global concern and a drive to reduce human exposure to MAP (FSAI, 2009). MAP has been cultured from pasteurized milk in the Czech Republic (Ayele *et al.*, 2005), UK (Grant *et al.*, 2002) and other countries such as Canada, USA, Argentina, Brazil and India (Chaubey *et al.*, 2017). MAP has also been found in Brazilian cheese (Faria *et al.*, 2014). This fact and the principle of precaution should encourage people to minimize human exposure to MAP by controlling Johne's disease at a farm level. Milk companies in some countries such as Denmark and the UK have started pressing veterinarians and farmers to implement control measures to reduce MAP prevalence on farms. This seems to be the sensible direction for both human and animal health and welfare.

## 1.3.4 Research and general public impact of MAP

Research has increased since MAP in humans  was first described in 1984 -
(Figure 1-5) sourced from PubMed (2018). This graph was obtained by searching
for: "*Mycobacterium avium* subsp. *paratuberculosis*" within the title, abstract or
body of the article. The total number of publications was labelled as "MAP
total". The filter "Human" showed the publications published on MAP in humans
and the filter "not human" showed the publications published on MAP in other
species. The most significant increases have been in the decades of the 90's and
2000's with a peak in 2011. From 2011 until now there has been a slight
decrease in research publications. The number of studies in animals, mainly
ruminants has always been higher than in humans. Although, research of MAP in
humans has been more consistent with around 35 papers per year.
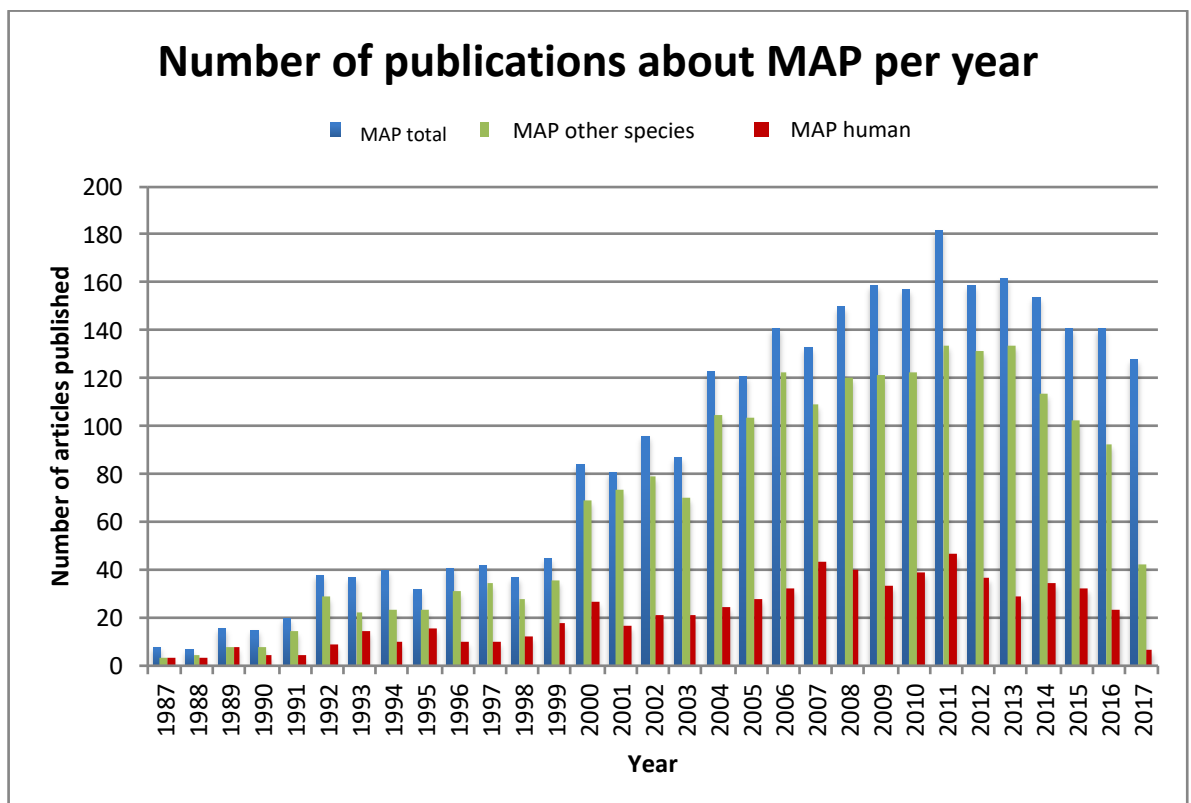


**Figure 1–4 Publications with *Mycobacterium avium* subsp. *paratuberculosis* on the title, abstract or body of the article.**

## 1.4 Pathogenesis and Time-Frame of MAP Infection

Infection with *Mycobacterium avium paratuberculosis* most commonly occurs by the faecal-oral route. Organisms shed in the faeces of infected animals are ingested by susceptible, usually young animals (<6 months old). Adults showing clinical signs of Johne's disease (weight loss and diarrhoea) are the main source of the bacteria, although infected animals without these signs can also shed the bacteria (Windsor and Whittington, 2010).

### 1.4.1 When does infection occur?

Paratuberculosis infection normally occurs in the first few months, or even first days of life, which means that young animals are the main source of newly infected individuals (Koets, Eda and Sreevatsan, 2015). This raised susceptibility to MAP in neonates has been attributed to various factors including increased intestinal permeability, a weakness of the mucosal barrier and impaired immunity. The rumen has also been hypothesized as protective in adult animals (Windsor and Whittington, 2010).

The new-born calf and its immediate environment can become contaminated with faecal MAP from an infected animal during the calving and post-partum period. The calving process, calving pen and colostrum have been thought to be the main sources of MAP for new born calves. Although, in the case of the colostrum it has been suggested that the main source of MAP in colostrum would be from the faecal contamination (>$10^7$ CFU/g) rather than actual MAP shedding from an infected cow in colostrum (100 CFU per 50/ml) (Sweeney, 1996; Caldow and Gunn, 2009). Thus, efforts in controlling hygiene at and around calving is seen as one of the key ways to control Johne's disease on farm (Sweeney, 1996; Clarke, 1997). Some research has suggested that calves can get infected from MAP in the environment outwith the immediate calving time. In high prevalence herds the risk of being infected with MAP was not higher in calves from positive cows (Eisenberg, Rutten and Koets, 2015) indicating that calves on these farms are infected via the environmental route rather than directly from infected mothers. The authors suggest that the environment is an important source of MAP on the farm and calves should be kept in specific areas with biocontainment measures enforced.

It has also been shown that calves that are exposed to a higher dose of MAP are more likely to become infected and they progress to the clinical stage more rapidly and have more severe lesions at PM than those ingesting a lower dose (Mortier, Barkema and De Buck, 2015). This would suggest that farms with a high MAP burden, due to the presence of super-shedders, (see section 1.6.3 – Super-shedders) high prevalence or poor biocontainment, are more likely to lead to infection in neonatal calves.

## 1.4.2 Additional sources of MAP in the farm

### 1.4.2.1 Colostrum and milk

MAP infected cows may also shed MAP in the milk and/or colostrum (Stabel, 2008), so consumption of these products by susceptible calves can also result in infection. Heavy faecal shedders are more likely to shed MAP and in higher quantities in colostrum than other MAP infected cows. Therefore, pasteurisation at 60°C for 60 minutes of colostrum and milk has been proposed in order to eliminate this source of MAP (Godden *et al.*, 2006). Colostrum pooling has also been identified as a risk as it can spread MAP to calves born from dams not infected by MAP. This has been described as a 'one to many' source of infection. Although, it is important to remember that shedding in milk and colostrum is relatively low compared to the MAP shed in faeces, thus hygienic colostrum collection is also of great importance.

### 1.4.2.2 Transplacental route

*In utero* transmission of MAP from infected cow to foetus has also been described and it is thought to occur on 20%-40% of the MAP-infected cattle (Clarke, 1997). According to Sweeney (2011) it is presumed, but not known for sure, that prenatally (*in utero*) infected calves will have a similar clinical progression and pathogenesis as calves exposed postnatally by the oral route. Although, this route of infection is not avoidable unless infected cows are culled and discarding offspring born to infected dams would be the only effective measure to control Johne's on farm.

### 1.4.2.3 Inhalation route

Trans-tracheal inoculation of MAP has been reported to lead to infection of calves. In this study (Eisenberg *et al.*, 2011) MAP was obtained by tissue culture from different parts of the intestine and draining mesenteric lymph nodes from calves infected by the trans-tracheal route. This finding suggests that MAP transmission by aerosol is possible and that, again, environment control may be important in order to minimise risk of infection of calves.

## 1.4.3 How does MAP infection occur?

The enteric route (specifically the ileum) is the most important portal for MAP infection. Specialized non-villous epithelial cells located in the Peyer's patches of the small intestine (which are present in high numbers in calves), known as M cells, identify MAP and facilitate translocation across the intestinal epithelium. Here, MAP organisms are phagocytosed by macrophages (Momotani *et al.*, 1988). Experimentally, MAP organisms were found within submucosal macrophages within 5 hours after direct inoculation into the ileum of calves (Momotani *et al.*, 1988). Despite the research reported, a minimum infective dose (MID) has not been established yet and it remains one of the significant knowledge-gaps currently (Barkema *et al.*, 2018). Most of the MAP experimental infections in calves are carried out with doses ranging from $10^7$ CFU (low dose) to $10^{10}$ CFU (high dose) (Sweeney *et al.*, 2006; Mitchell *et al.*, 2012; Rienske, Mortier *et al.*, 2014; Corbett *et al.*, 2017) Although, the lowest dose of MAP ever described to cause clinical disease in ruminants was of $10^3$ CFU (Begg and Whittington, 2008).

The first time a pathogen encounters the host's immune system, macrophages are activated through the innate immunity pathway. This strong inflammatory response mediated by T-helper type 1 cell (Th1) and type M1 macrophages (cellular immunity response) is the typical first immune response observed when MAP infection occurs. If a Th1 response predominates, lymphocytes will produce interferon-gamma and other cytokines which will enhance the killing of intracellular MAP organisms. In this case, MAP organisms would be eliminated and thus, infection would not progress to clinical Johne's disease (Mortier *et al.*, 2015; Begg *et al.*, 2018).

In most cases calves are able to control the early infection (Mortier *et al.*, 2015). Although, the immune response is not entirely efficacious due to MAP organisms having the ability to impair phagocytosis by the macrophages which allows the MAP to slowly proliferate and spread within the gut and gut-associated lymphoid tissue (Sweeney, 2011). During this time, the animal does not show any clinical signs. However, as the infection slowly progresses, MAP manages to disseminate and the lesions become more severe, eventually leading to the clinical signs of Johne's disease.

## 1.4.4 Infection of adult cattle: less frequent but still possible

Espejo *et al.* (2013) found that adult cattle can become infected and develop clinical Johne's disease signs. This feature has also been described by other authors (Windsor and Whittington, 2010; Mortier *et al.*, 2013). Although adults can be infected, they need a highly contaminated environment and are less likely to develop clinical paratuberculosis compared to calves. This highlights the importance of not only controlling Johne's infection of calves but adults also.

Becoming infected as an older animal could be of relevance in herds where adult cows graze in the same field as breeding heifers. Dairy farms in the UK are increasingly reducing grazing time for high yielding dairy cows, but the animals that are often still turned out for grazing are heifers, dry cows and low yielders. Dry cows (close to calving and more prone to shed MAP) and low yielding cows (that could be infected and shedding MAP) have the potential to shed MAP and contaminate pasture (see section 1.4.2 Environmental persistence). Heifers could potentially get in contact with MAP from the pasture and intake a significant amount of MAP if grazing these areas (Mortier *et al.*, 2013).

## 1.4.5 Progression to clinical disease

After an indeterminate time period (which varies between individuals) the infection can no longer be controlled by the host immune system. The trigger of this moment is not yet fully understood, but it seems that a stressful event (such as calving, parasitism or other concomitant diseases) leads to further decrease of the cell-mediated immunity and MAP infection progresses. Then, there is a

transition from a Th1-type immune response towards a Th2-type immune response (humoral based response). When this occurs, infection within the gut progresses more rapidly and the animal starts showing clinical signs. Antibodies are not protective as MAP is an intracellular bacteria. Hence, MAP can multiply without any effective immune response and is shed in significant numbers in the faeces (Koets, Eda and Sreevatsan, 2015).

With time, the inability to control the infection leads to a worsening of the clinical signs and the amount of MAP shed in faeces increases. Death of MAP infected macrophages in the lamina propria of the intestine leads to free MAP in that area; MAP will be taken up by dendritic cells migrating to the lumen, or excreted via fluid streams leading to MAP shedding in faeces (Koets, Eda and Sreevatsan, 2015). However, the course of the disease and immune status are variables that will make faecal shedding vary between individuals (see section below). Lesions in the intestines and mesenteric lymph nodes become more severe, diffuse and extensive and clinical signs (diarrhoea and weight loss) become evident.

## 1.4.6 MAP shedding cycle

The MAP shedding pattern in cows has been subject of different studies. Laurin *et al*. (2015) identified different aspects that play a role in the shedding pattern of cows. Such factors were days in milk (DIM) and season (Figure 1-6). Probability of a positive PCR result was most likely in freshly calved cows (due to immunosuppression) and in winter time (possibly related to housing and increased stocking density).
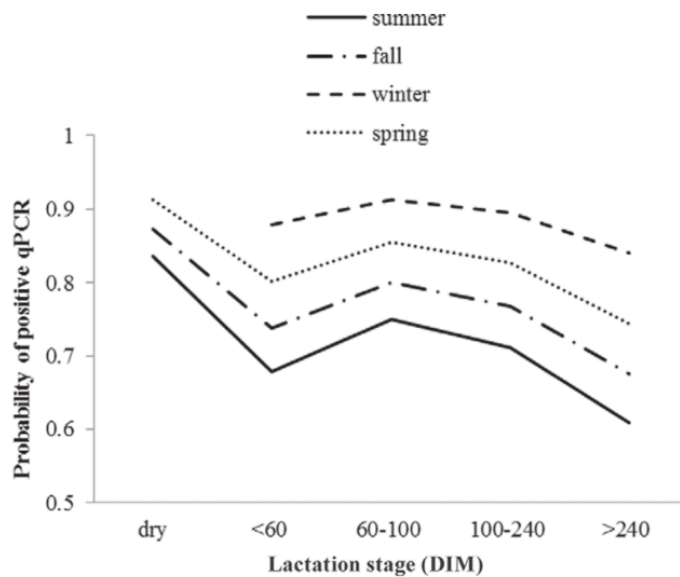
**Figure 1–5 Predicted probability for detecting MAP by PCR on faeces (Laurin *et al.*, 2015).**

Koets *et al.* (2015) suggested that a timeframe of 5-6 weeks is the time it takes 1CFU (colony forming units) of MAP to replicate and shed 300-500 MAP. In this article it is suggested that strategic repeated faecal samples could improve the sensitivity of MAP shedding animals by PCR or culture Koets et al. (2015).

### 1.4.6.1 Shedding pattern in calves

Shedding of MAP in calves has been proven by different studies (Mortier *et al.*, 2014; Wolf *et al.*, 2015). Such investigations provided evidence that naturally infected calves can shed MAP and also infect other calves.

## 1.4.7 Liver fluke role in MAP infection

Liver fluke is a very common disease of grazing cattle with a worldwide distribution (Howell *et al.*, 2015). Recent studies have suggested that *Fasciola hepatica* could accelerate development of Johne's disease and appearance of clinical signs. *F. hepatica* infection reduces cell mediated immune responses, polarising host's immune response towards a humoral immunity, this increases the host's susceptibility to many bacterial, viral and protozoal pathogens (Naranjo Lucena *et al.*, 2017).

Naranjo *et al.* (2017) reviewed the pathogenesis of mycobacteria in cattle and suggested that Bovine Tuberculosis (BTB) infected animals that show false negative results to intratuberculin and Interferon gamma (IFN-$\gamma$) tests could be attributed to F. *hepatica* due to suppression of cellular immune response. Due to similarities between BTB and MAP host immune response it is possible that F. *hepatica* could supress cellular immune response in MAP infected animals and lead to a clinical phase of Johne's disease. Although, Naranjo et al. (2017) hypotheses this causal effect lacks evidence and recognises a specific study should be carried out to elucidate this issue.

## 1.4.8 Genetic susceptibility

It has been reported that the Channel Island breeds of cattle (Guernsey, Jersey) have a higher prevalence of Johne's disease compared to Friesian or other breeds. Thus, it was speculated that those breeds were more susceptible to MAP infection. However, it is not possible to determine whether these anecdotal observations represent a true genetic effect on susceptibility and to what extent management, geography, and other influences confound the observation. (Kirkpatrick and Shook, 2011; Sweeney, 2011). A single study reported an increased prevalence of Johne's disease in Limousin animals (Richardson and Ekb, 2009), this study was carried out in Cork and proposed infected Limousin sires as an important factor for the spread of Johne's disease in that area. Some studies have pointed to the lower prevalence of Johne's disease in beef herds as a potential indicator of resistance to MAP, but it has to be taken into account that the extensive system that is normally used in beef production is probably the most important factor (Barkema *et al.*, 2018).

In another paper, Koets *et al.* (2010) determined a single nucleotide polymorphism (SNP) linked to Johne's disease susceptibility. Animals with this particular SNP may have less resistance to MAP. Oher studies link polymorphisms in TLR-4 (Sharma *et al.*, 2015) and TLR-1(Cinar *et al.*, 2018) as possible genes linked to increase resistance to MAP. Breeding for genetics that did not contain this SNP could allow selecting animals with better resistance to MAP.

# 1.5 Clinical Signs and MAP clinical presentations

Johne's disease is an insidious and chronic condition characterized by weight loss and diarrhoea, where clinical signs establish gradually. Initial clinical signs are mild and non-specific and it is difficult to establish the exact onset of clinical disease. Time between infection and first clinical signs varies a lot between individuals, but in most cases, cows develop clinical signs between two and five years old (Nielsen and Toft, 2006).

An innovative and comprehensive body of work has been performed in order to define the different stages of the disease and a case definition has been proposed by Whittington *et al.* (2017). This paper is the most complete attempt to describe all the different outcomes that have been reported in relation to MAP infection. This should help to classify MAP infected animals properly in relation to their clinical signs and test results into different stages or categories of infection. The relevant parts of Whittington's definitions are presented below as a guide.

## 1.5.1 Sub-clinical stage

At this stage, the animal is infected but does not have clinical signs attributable to paratuberculosis as the animal's immune system stops MAP proliferation. These sub-clinically infected animals may have more subtle signs of illness such a low body condition score, low milk yield and failure to get back in calf, but this signs cannot be attributed to Johne's disease *per se* (Whittington *et al.*, 2017). They often go unidentified in farms where testing of only animals showing clinical signs is carried out. Herd sampling strategies that involved screening all animals over multiple time frames may identify these animals.

## 1.5.2 Clinical stage: Johne's disease

Clinical signs of Johne's disease may appear following stressful events, such as calving, environment changes, changes in the feeding ration, metabolic challenges (early lactation) or co-infection with other agents such as liver fluke (Naranjo Lucena *et al.*, 2017).

It has been shown that for every cow showing clinical signs, there is between 15-20 additional infected animals within the herd (Whitlock *et al.*, 2000). Although, according to the same author, only half of them would be identified by faecal culture. Thus, animals showing clinical signs suggestive of Johne's disease should always be tested in order to identify if Johne's disease is present in the herd and (if the animal is positive) remove a potential source of MAP in the farm. Moreover, if Johne's disease is confirmed in the farm, additional testing and control measures should be put in place (see Section 1.8-Diagnostic tools).

The typical clinical signs of Johne's disease in cattle are watery ("pipe-stem") diarrhoea and weight loss. This diarrhoea, which can be intermittent, is due to a malabsorption caused by the granulomatous inflammation of the intestines. Such condition induces the progressive emaciation of the animal. It is worth remarking how the cow's appetite usually remains good until the terminal stages of the disease (Whittington *et al.*, 2017). Given that clinical signs of paratuberculosis are not specific, Whittington *et al.* (2017) has recently proposed a case definition for the clinical stage as: demonstrable weight loss, measured as ≥10% body weight loss over one month and/or low body condition score compared to the majority of animals in the herd.

At the terminal stages, the clinical picture of affected animals is cachexia and dullness. In some animals, appetite can be reduced and other conditions such as mastitis, endometritis, pneumonia and lameness can also be present. In some animals, the significant hypoproteinaemia can cause subcutaneous oedema, normally located in the brisket and submandibular region (colloquially called bottle jaw) (Whittington *et al.*, 2017) .

## 1.5.3 Super-shedders

Cattle classified as super-shedders are animals infected with MAP in which the infection is no longer under control within the animal. These animals, with some exceptions, are normally at the end-stage of Johne's disease and clinical signs are normally obvious and severe (Sweeney, 2011). Animals classified as super-shedders are the ones that shed more than 10,000 UFC/g of faeces, although some animals have been detected shedding 1,260,000 UFC/g of faeces (Aly *et*

*al*., 2012). This means that one gram of faeces of a super-shedder could potentially infect 1,000 calves.

One study tried to identify these animals by different methods (Aly *et al*., 2012). The most cost-efficient method used faecal PCR CT values on the animals that previously tested ELISA positive (milk samples for milking cows and blood samples for dry ones). This strategy costed $1,230/super-shedder identified and had a sensitivity of 47% (Aly *et al*., 2012). In several studies PCR CT values have been used in order to estimate the amount of MAP shed by a cow, despite the correlation seen, there is still no method that can predict with exactitude the CFU shedding of an individual cow (Aly *et al*., 2012; Laurin *et al*., 2015). The only method to quantify the amount of MAP shed by an animal is diluting the sample and counting the CFU after culture, which is laborious, time-consuming and just applicable to research studies (Whittington, 2010).

## 1.5.4 Additional clinical presentations hypothesized

Regarding the complexity of the disease and the difficulty in classifying all individual cases within the definitions described above, Whittington *et al*. (2017) proposed two new concepts: resistance to MAP and recovery from MAP infection. Neither of them have been demonstrated, but they are still conceptually possible and they appear in other research papers.

- Resistance to MAP: animals that are known to have received an infectious dose of MAP at an age when they were susceptible but infection does not establish (Whittington *et al*., 2017).

- Recovery from paratuberculosis: elimination of a demonstrable infection. Proof of recovery would require detailed examinations at more than one time point. Recovered animals may have residual histopathological lesions, but the lesions should be mild or of a lower grade than those observed at an earlier time point (Whittington *et al*., 2017).

## 1.6 Diagnostic Tools

As it has been described above, clinical signs of Johne's disease are non-specific. Thus, to reach a definitive diagnosis, a positive laboratory test is required. The issue with the available laboratory tests are the low sensitivity and the inability to detect infected animals in early stages of infection. It is important to understand the pathogenesis and immune response in order to interpret the test results properly. This can be challenging due to the nature of the disease.

In this section, the different available paratuberculosis laboratory tests will be described, pointing out the strengths and limitations of each test as well as interpretation of the result.

### 1.6.1 ELISA testing

This test is an absorbed indirect ELISA (Enzyme-linked immunosorbent assay) that detects IgG1 (Immunoglobulin G1) antibodies against MAP. Due to the delayed humoral immune response against MAP (humoral immunity develops 10–17 months after infection) the sensitivity of serum (and milk) ELISA in subclinical animals is low (around 15%) but much higher (around 90%) in clinically affected cattle (Whitlock *et al.*, 2000).

Because MAP infections are chronic but progressive, the sensitivity and specificity of serology increases as the disease progresses and as the animal gets older (see Figures 1-7 & 1-9). As a result, this test is normally used only in animals over two years old and/or in animals presenting with clinical signs. This poses a problem in herds that buy in replacement heifers (normally younger than two years old). Ideally sampling these individual animals would be desired but as antibodies would likely not be detected it is recommended to buy animals from accredited low risk farms. Therefore, instead of testing individual bought in animals the most valuable information comes from serology results from all cows on that herd over a historic time frame (Cashman *et al.*, 2008).

In the field, serology results can be interpreted alongside milk yield data and this can be a useful predictor of being infected with MAP. A positive ELISA result in a cow experiencing low milk yield for stage of lactation will have a higher

predictive value than the ELISA result alone (Nielsen, 2009). As a consequence, combination of repeated ELISA and evaluation of milk production can be a practical tool to base decisions for culling. It is also worth to remark that positive predictive value (PPV) and negative predictive value (NPV) of any test varies depending on the prevalence of a disease. In this case, PPV of serology in farms with a high prevalence increases. Similarly, a NPV in farms with a low prevalence will increase. Clinicians should be aware of the prevalence in the region and estimate the prevalence on the farm where no data is available. This is key in order to interpret serology results from the laboratory (Caldow and Gunn, 2009).

Serology is a test of a high value in cows showing clinical signs compatible with Johne's disease (Figure 1-9). The sensitivity in these animals is reported to be 99%, which means that a positive result on an animal with weight loss and diarrhoea provides a very high PPV that cannot be improved by any additional testing (Weber *et al.*, 2009).

Serology performed in calves inoculated with MAP was carried out by Mortier *et al.* (2015) and showed positive results in young calves. The chance of detecting a MAP infected animal in the early stages of infection was highest in the 6 months following infection. These results suggested testing calves as well as adult cattle during screen testing to detect MAP infected animals as early as possible. Mortier *et al.* (2015) stated that early diagnosis could be achieved by combining ELISA and faecal culture in young stock. Although, there was no information about maternal derived antibodies and the interference this could have.

### 1.6.1.1 Influence of tuberculin test

It is reported that the Intradermal tuberculin test (IDTT) can interfere with Johne's serology, leading to false positive results. Kennedy *et al.* (2014) studied these interactions and concluded that IDTT can influence Johne's serology for up to 71 days in case of blood ELISA and 43 days in case of milk ELISA. After this period of time, antibodies dropped back to pre-IDTT levels. As a consequence, sampling for Johne's should not be carried out before the described days have elapsed following IDTT testing. Although, it is worth mentioning that some

diagnostic laboratories recommend a period of three months between IDTT and a Johne's serology test (Anonymous, 2017).

On the other hand, it could be hypothesized that IDTT might act as a "booster" in MAP-infected animals and increase IgG1 response towards MAP. Infected animals that had not yet seroconverted would start producing anti-MAP antibodies after IDTT. As a consequence, serology after IDTT would allow identification of MAP-infected animals before they would normally be serology positive. This is supported in the literature; Kennedy *et al.* (2014) hypothesized that the tuberculin test might act as a vaccine with cross protection against MAP, which would diminish clinical signs and production losses of the animals. In conclusion, interactions between IDTT and Johne's disease is an area for further research. There could be interesting avenues to explore in terms of increased serology sensitivity leading to earlier diagnosis of the disease.

### 1.6.1.2 Blood serology

This test is performed on a serum sample. This test is probably the most used laboratory test on an individual animal basis after a suspicion of Johne's disease. It is cheaper and it has a faster turnaround time tests than other tests (Figure 1-9). Its specificity is very high, around 99% (Weber *et al.*, 2009), which means that less than 1% of positive results are a false positive. Moreover, some studies have shown a correlation between ELISA percentage of positivity and magnitude of CFU faecal shedding (Collins, 2002). This last feature could help farmers to cull presumed high shedders based on serology percentage of positivity basis by culling first, cows with the highest % of positivity.

This test is commonly used in beef herds that test on a yearly screening basis and in cattle showing clinical signs to confirm a suspected case (Collins, 2011). Although, dairy farms prefer to use milk serology as it does not require a veterinarian to take the sample and the test can be done on the milk samples obtained regularly for the milk record.

### 1.6.1.3 Milk testing

This test is performed in an individual milk sample, but it has a lot of similarities with blood serology test. The obvious difference is the less invasive sampling

collection and ease of sampling (which can be done by the milker during milking routine or by the milk recorder and doesn't require a veterinarian). Milk testing can be performed on milk samples taken for milk quality testing, which many dairy farms already do and negate the need to additional sample collection. This test has great possibilities and allows testing of the whole milking herd in a cost-effective manor, it would not be suitable for a beef herd however. Although it has been reported to have a slightly lower sensitivity and specificity than blood ELISA, milk testing is commonly used on a quarterly basis in dairy farms that want to control and monitor Johne's status (Anonymous, 2017).

Nielsen (2009) has studied milk testing extensively as part of the Danish national Johne's control program. This author stated that repeated milk testing is a valuable tool in Johne's disease control programs on farm. In different papers he dissected the main benefits and features of performing this test on a regular basis. He stated that most animals shedding MAP will develop antibodies at some point (Nielsen and Ersbøll, 2006) and that ELISA positive animals have higher probability to be bacterial shedders (Nielsen, 2008). An important feature of the milk ELISA was that 70% of high shedders tested positive on ELISA on the date that bacterial shedding started. Consequently, a positive ELISA result could predict, even before any faecal shed, an animal that would subsequently become infectious (Nielsen, 2008). This statement contradicted previous research, which affirms that shedding occurs before the animal seroconvert (Whitlock *et al.*, 2000) . Only 5% of transient shedders were ELISA-positive on the date of first detected bacterial shedding (Nielsen, 2008), which means that some infectious cows will remain undetected after faecal shedding onset (Figure 1-7).

Considering that antibody response fluctuates over time (Figure 1-7), ELISA value has to be interpreted as a probability of MAP infection and shedding rather than a yes/no answer. Thus, frequent milk testing allows detection of MAP-infected cows earlier and this will allow farmers to cull MAP shedders. Detection of MAP-infectious animals appears to be affected more by test frequency than it is by age (Nielsen and Toft, 2006).
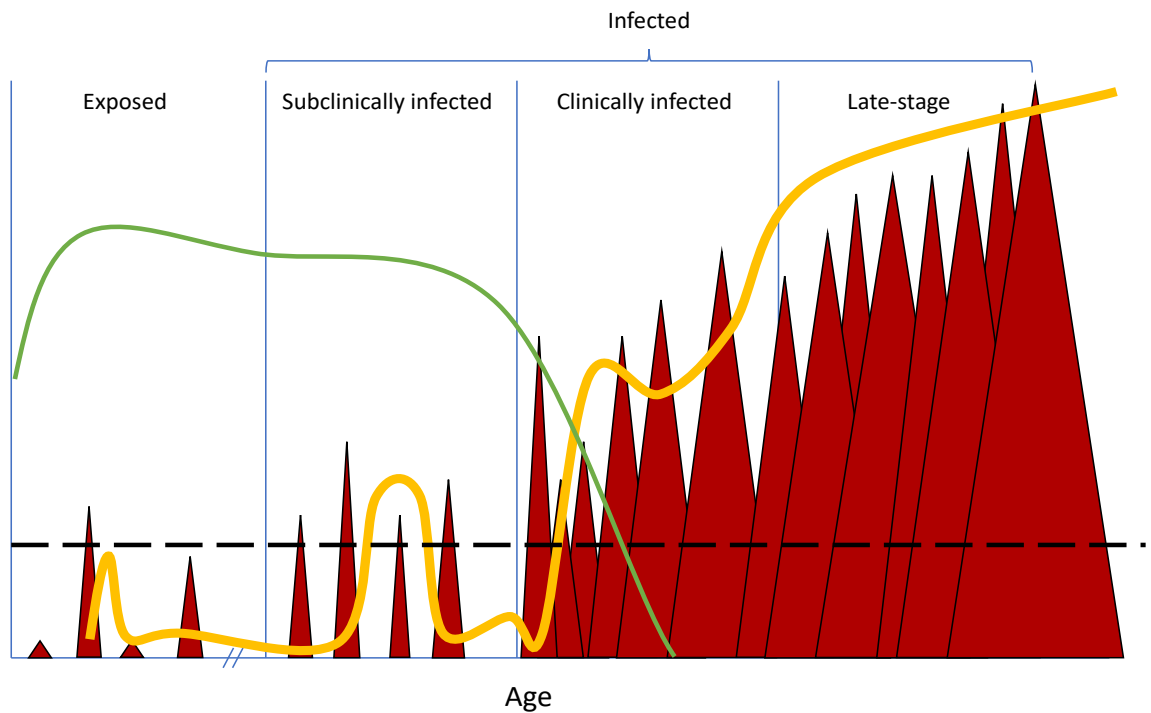
**Figure 1–6 Graph showing the typical progression of a MAP infection in four different stages. In green – cellular response, in yellow – humoral response, in red – bacterial shedding in faeces, discontinuous black line marks the threshold that would allow detection in serology (yellow line) or faecal culture (red). Adapted from (Nielsen, 2008; Rienske A R Mortier et al., 2014[b]; Koets, Eda and Sreevatsan, 2015).**

Eisenberg *et al*. (2015) found that when sampling healthy dairy cows on a monthly basis, colostrum ELISA yielded more positive results than milk ELISA. In other words, as days in milk increased, the ability to detect MAP antibodies decreased. Sensitivity and specificity of colostrum testing was not calculated, but in this study, MAP specific antibodies were present in 34% of the colostrum samples, whereas milk testing was positive only in a 11% of the cases. Despite the promising advantages of colostrum ELISA against milk ELISA, there is still work to be done. Follow-up of ELISA positive cases should be carried out and a comparison with faecal culture and histopathology should be carried out.

Milk testing can be applied in various ways on farm depending on budget, prevalence and ultimate aims of the Johne's control programme. There are two main options:

1. Quarterly individual milk test. Following a positive serology, the farmer can then decide what to do given the history and previous test results of the cow

    a. Multiple positive tests (consecutive or not) indicate a very high probability of the cow to be MAP-infected. Cull is advised.

    b. A positive test followed by a negative result indicates a cow with high probabilities to be MAP-infected. Further testing with blood serology, faecal PCR or culture can be performed to confirm disease.

    c. A positive test followed by multiple negative results can indicate a false positive (<1% of cases), recording mistake or a late sero-converter. True false positives are thought to be commonly related to labelling mistakes in the milking parlour (Helen Carty personal communication). Other tests can be performed if needed.

2. Single milk test. Studies in order to maximise benefit of a single test have been carried out, these suggest that the best time to do a single milk test would be at the end of lactation (Eisenberg *et al.*, 2015). If there is a positive result, it can be confirmed by individual blood serology or faecal PCR. Decision on rebreeding can be made before the subsequent lactation starts. Calving and colostrum management of positive cows can also be changed depending on results from this test (Eisenberg *et al.*, 2015).

**1.6.1.4 Bulk Milk ELISA**

Bulk tank milk ELISA is performed on a bulk milk tank sample of the farm. It has been demonstrated to have a low correlation with herd prevalence based on individual milk ELISA (Nielsen and Toft, 2014). Therefore, bulk milk antibody ELISA testing is not recommended as a suitable tool for indicating prevalence of Johne's within a herd. A low result is misleading because bulk milk result can be low even if 9-12% of the animals contributing to the tank are seropositive for the disease (NMR, 2011).

## 1.6.2 Interferon gamma (IFN-γ)

This test measures the release of IFN-γ by lymphocytes after in vitro stimulation with Johnin PPD (purified protein derivate) antigen or M. *bovis* PPD antigen. It is performed in a blood sample and the test needs to be performed within 12h from sampling. It is a good indicator of the cellular immune response that occurs shortly after MAP infection and is present during the subclinical stage of infection.

Authors have found IFN-γ test an additional useful tool. The result of IFN-γ, which peaks 4 months after infection, could be used on farm to detect calves that have been in contact with MAP (Mortier *et al.*, 2014[a]). In fact IFN-γ would be a better indicator of early stage of a MAP infection. (Mortier, Barkema and De Buck, 2015), which could allow farmers to take a decision on animals before rearing them. Despite its promising benefits, it has been reported to have low sensitivity and specificity (Huda, Jungersen and Lind, 2004).

To the author's knowledge, this test is only used in research. It is not available in any of the commercial veterinary laboratories in the UK and the cost and the low specificity of the test are probably the main reasons that currently impair its use.

## 1.6.3 Culture

This laboratory test detects MAP growth by incubating the sample in either solid or liquid selective media. There are different culture media that can be used, but the Herrold's egg yolk medium (HEYM) and modifications of it are the ones that are most commonly used. Due to the slow growth of MAP, time to grow the bacteria normally takes six weeks at 37°C. After this period, it is recommended to perform a PCR of the colonies grown or on the culture liquid to confirm that the bacteria isolated is MAP.

Among all detection methods of MAP, culture has historically been designated the gold standard (Whitlock *et al.*, 2000; Nielsen *et al.*, 2005; Metre *et al.*, 2008). The highest sensitivity amongst all the tests in the live animal and a specificity assumed to be 100% are the main reasons that make this test the

"Gold standard". The main down side of this test is the long time period to yield the result due to the slow growth of the bacteria, which takes at least 42 days. It can detect animals shedding more than 100 colony-forming units per gram (CFU/g) of faeces (Rideout *et al.*, 2003), but does not detect any of the "MAP-infected, not MAP shedding" animals (see section 1.6.1 Subclinical stage). This is the reason why, despite being the "gold standard test" the sensitivity is far lower than we would desire when compared to other diseases.

Sensitivity of MAP culture has been reported to range from 33% to 70% (Whitlock *et al.*, 2000; Nielsen and Toft, 2008). Numerous studies have been performed in order to establish the sensitivity of culture and outcomes are very variable. Differences in reported sensitivity values are mainly due to the study design. The main differences noticed between studies are the length of time elapsed for follow up testing and the age of the animal at time of sampling. As a result, studies that follow up the cases for a long time period will be able to detect a higher number of animals that in other studies where positive cases would be missed. Thus, it is difficult to determine an accurate value since culture results can only be compared to culture results obtained later on the same animal (Whitlock *et al.*, 2000; Nielsen and Toft, 2008).

The main reason of this impaired sensitivity for culture is the variable faecal shedding of MAP in cattle (Figure 1-7). Given that all infected animals that are shedding at the time of sampling will be positive for MAP culture, all false negatives will come from faecal samples taken during non-shedding periods of infected animals (Whitlock *et al.*, 2000). Non-MAP-shedding but infected animals will be young animals and intermittent shedders (Nielsen and Toft, 2008), which explains why culture sensitivity in young animals is lower than in adults (Figure 1-9). In this study and its literature review sensitivity and specificity values of the different tests are exposed as reported in the scientific papers. However, it is also important to highlight that other test sensitivities are normally calculated in comparison to culture (Whitlock *et al.*, 2000). This implies that sensitivity values of other tests need to be weighted in relation to culture values (Whitlock *et al.*, 2000). In other words, sensitivity values of other tests cannot be higher than culture. All values of sensitivity and specificity should be interpreted

carefully and it should be acknowledged that comparing other tests to a test that doesn't have a 100% sensitivity and specificity is not ideal (Table 1-1).

The age at which animals are recommended to be sampled for Johne's culture is over two years of age. Although, Mortier *et al*. (2015), suggested that culture testing calves could be beneficial in herds where there is the intention to control Johne's disease. Data from this study showed 61% of calves tested two weeks after inoculation had positive culture results. In this study, calves were infected with low or high dose of MAP at two weeks, 3, 6, 9, or 12 months of age. Earlier diagnosis could be achieved by combining ELISA and culture in young stock (Mortier, Barkema and De Buck, 2015). Although, it might be an expensive strategy, this approach could be key in farms trying to grow calves "likely MAP-free". One criticism of this paper is the infection model of the animals. Given that a minimum infectious dose has not been yet established for MAP (Barkema *et al*., 2018), this study should have stated CFU used so that future studies can replicate it, use other infection doses and most importantly allow comparison between infectious doses. It could be argued that natural infection could lead to a different infection process and different shedding pattern that the one observed in this study. In another study, Eisenberg *et al*. (2015), described that in infected animals there is a MAP shedding peak between 7-14 months, which could be a useful timeframe to consider when performing culture testing in order to try to detect positive heifers before deciding to breed from them.

Culture testing can also be used in pooled faecal samples. It has been shown that pooling five faecal samples in one pot can still detect 88% of samples that contained one positive animal (Kalis, Barkema and Hesselink, 2000). This sampling method could be used in order to decrease costs and its sensitivity is not significantly different from an individual culture test. Positive pools would then need to be further sampled at an individual cow level to detect which animals in the pool were in fact infected. Quarterly serology testing would still be cheaper than pooled culture testing however (Table 1-1).

Environmental culture testing has been performed at a farm level in order to detect Johne's positive farms. In one study, samples from cow alleyways, manure storage, calving area, sick cow pen, water runoff, and post-weaned calves areas detected Johne's disease in 78% of the herds sampled (Raizman *et*

*al.*, 2004). A positive farm was defined as one or more positive results in the areas sampled. The number of positive environmental samples also correlated with herd prevalence. Thus, composite environmental faecal samples can be the first step to detect herd infection.

In conclusion, although culture is not broadly used in field conditions due to the time taken to grow the bacteria, it is the gold standard method to diagnose paratuberculosis and it is still crucial for confirmation of MAP-infected animals and key in most research projects.

## 1.6.4 Polymerase-chain reaction (PCR)

MAP detection by PCR is a direct diagnostic test that identifies MAP DNA (deoxyribonucleic acid) within a sample. Normally used in faecal samples, this test detects faecal shedding from individual animals, which occurs before the antibody response (Figure 1-7). This allows infected animals to be detected before they test positive on serology and before they show clinical signs (Figure 1-9).

The main advantage of the PCR technique is that it has a fast turnaround time of two days (Table 1-1), which is much faster than culture (42 days). In addition, it is also less expensive than culture (Table 1-1). As an organism-detection test, PCR gives definitive identification of MAP in a sample. There are no interferences or crossed-reactions as in the case of serology and TB testing. In addition, it is a versatile test that can be performed in individual, pooled or environmental samples in order to reduce costs. PCR can also be performed in milk or tissues (normally from post-mortem examination) (Collins, 2011).

Detection of MAP by PCR does not confirm the presence of an active infection, which leads to some false-positive results due to gastrointestinal "pass-through" effect (Hines *et al.*, 2007). No more does faecal culture for the same reason (pass through). In herds that are heavily infected with MAP, PCR results should be interpreted carefully. In these cases, a pass-through of MAP (rather than active shedding) is more likely to occur. It is important to note that this test is still more expensive (approximately by six times) when compared to ELISA (SRUC, 2016).

Reported performance of this test varies a lot. Sensitivity can range from 18 to 94% and specificity ranges from 86 to 100%. The main cause of differences in performance is the ability of the tests to overcome the components in faeces that inhibit the PCR reaction (Leite *et al.*, 2013). Accuracy of test and result interpretation will depend on the PCR kit and DNA fragment targeted by the PCR kit used by the laboratory of reference in a particular area. The two main DNA sequences targeted are IS900 and ISMAP02. It is also worth mentioning that due to the lack of knowledge about shedding patterns there is no recommended time of re-sampling (as it also happens with serology) to optimize MAP detection, this is still one of the major knowledge gaps that potentially impairs the best use of MAP PCR and culture tests (Barkema *et al.*, 2018).

### 1.6.4.1 Cycle threshold (CT) values

PCR allows quantification of the amount of MAP in a given sample by the count of cycle threshold values that a sample needs to have detectable MAP DNA. This means that an indirect quantification of MAP can be inferred by the CT value of a sample. In this case, the lower the CT value is, the higher the quantity of MAP there is in a sample. It has been observed that moderate or light shedding categories generally corresponded to qPCR (Quantitative PCR) cycle threshold values >35, but heavy shedding categories corresponded to qPCR values <29 (Laurin *et al.*, 2015).

Consideration of CT values could be used in MAP control programs in a farm. Knowing that lower CT values are indicative of greater MAP bacterial load in a cow's faeces (Laurin *et al.*, 2015) could lead to prioritizing culling or management of high-shedding cows. This would be very relevant in high prevalence herds, where not all positive cows can be culled immediately.

In conclusion, PCR is a valuable test with potential to be used in cases where the clinician suspects MAP infection is probable (i.e. progeny of a positive cow). In addition, it is largely used also after a serology positive test in cases where the cow has no clinical signs and the clinician suspects that it could be a false positive (i.e. herd tested negative the last two years).

## 1.6.5 Gross pathology

Gross pathology examination aims to identify gross lesions that are commonly described in animals suffering from Johne's disease. Lesions associated to Johne's disease are the thickening and corrugation of the mucosa of the intestine (typically localised in the terminal ileum), enlargement of the mesenteric lymph-nodes and lymphangitis (Figure 1-8). Atrophy of the mesenteric fat, subcutaneous oedema and mineralisation of the aorta are other findings that have been reported in animals with Johne's disease.
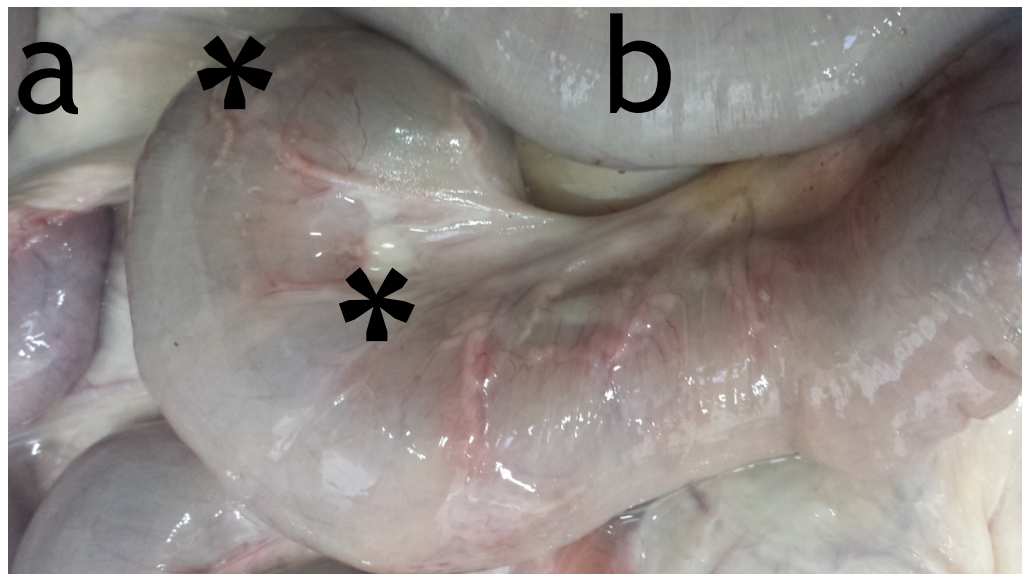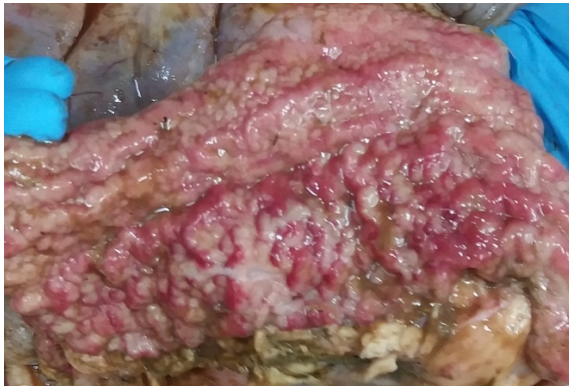


**Figure 1–7 Lesions suggestive of Johne's disease. a- corrugation and thickening of the mucosa of the ileum; b- mesenteric fat atrophy and lymphangitis; c- detail of lymphangitis (\*) lymphatic vessel. Joan Baró - University of Glasgow.**

Gross examination is an important part of the diagnostic process in individual farm animal cases. It provides an immediate overview of the health status of the

diseased animal and it is particularly important in animals that die suddenly and without any clinical or ancillary test information (Caldow and Gunn, 2009). The post-mortem examination (PME) is also a crucial opportunity to take the samples that are needed either for histopathology, tissue PCR or tissue culture and key to rule out other pathologies on an animal with suspected Johne's disease.

Zeihl-Neelsen stain (ZN) can be also performed in impression smears from tissues and/or faeces of the animals suspected to suffer from Johne's disease. It is a specific test that provides a fast answer. However, several studies report a low sensitivity for faecal smears of 43% in animals showing clinical signs (Weber *et al.*, 2009).

Despite the invaluable information that PME provides, there is a lack of reported research into gross pathology as a diagnostic tool for Johne's disease (Flook, 2015). Therefore, no sensitivity or specificity have been studied or proposed. Although, different authors have proposed PME as an appropriate tool in the clinical and end-stages of the disease, when the lesions would be clear and with a limited accuracy (Whittington *et al.*, 2017). Thus, it is always advised to sample any suspicious lesions and confirm the aetiology with further analysis of the samples such as tissue PCR/culture or histopathology.

## 1.6.6 Histopathology

Histopathology identifies lesions produced by MAP in tissues sampled from infected animals. This technique is normally performed by biopsy through rectal endoscope in humans for diagnosis of Crohn's disease, but due to the difficulty of the technique and ethics of the procedure this is a test that is normally done after a post-mortem examination in cattle. Sampling sites are normally intestines and or mesenteric lymph nodes showing gross pathology. Further processing of the samples consists of fixing with formalin, cutting, making the paraffin block and staining has to be carried before microscopic examination. The two main stains used to diagnose Johne's disease are haematoxylin eosin stain (to identify lesions consistent with Johne's disease) and Ziehl-Neelsen stain (stains with a bright magenta colour the Mycobacteria present in the tissues). A combination of microscopic pathology and identification of MAP within the

lesions lead to a positive result. Thus, it is assumed that specificity of this test is of 99%.

Sampling site for histopathology has been discussed and varies between authors, but the ileo-caecal valve and adjacent lymph node have been identified by many article reviews as the preferred site of sampling (Whittington *et al.*, 2017). Histopathology exam (which normally include H/E and ZN stained slides) is more sensitive and specific than gross pathology, which can miss early and subtle infections and also can mistake other similar intestinal lesions (i.e. eosinophilic enteritis). Histopathology examination is also confirmative for the cases where gross lesions are dubious or even in cases where lesions are highly suggestive of Johne's disease, but no other test has been performed and a confirmation is needed. It has been shown (Martinson *et al.*, 2008) that specificity is very high, around 95% (other non-pathogenic mycobacteria can potentially be found). Although, sensitivity is thought to be low. In a study just 9% of the cows (7/78) that had a positive result in tissue culture were identified by ZN stain examination (Martinson *et al.*, 2008). Other studies have reported different values for histopathology sensitivity, which ranged from 0% to 100% (Huda and Jensen, 2003; Sweeney *et al.*, 2006; Martinson *et al.*, 2008). These differences reported are due to the study design of each paper (see Chapter 4 Discussion – section 4.6 Histopathology).

| | Exposed | Subclinically infected | Clinically infected | Late-stage infected |
|---|---|---|---|---|
| PCR/Culture | 🟧 | 🟧 | 🟩 | 🟩 |
| ELISA | ⬜ | 🟧 | 🟩 | 🟩 |
| Post-mortem | ⬜ | ⬜ | 🟧 | 🟩 |
| Histopathology | ⬜ | 🟧 | 🟩 | 🟩 |

**Figure 1–8 Schema of the different tests and their sensitivity in different stages of the disease. Grey (poor), orange (average), green (good). Adapted from (Nielsen and Toft, 2008; Whittington *et al.*, 2017).**

### 1.6.7 Future of MAP laboratory tests

As discussed above, none of the laboratory tests available for Johne's disease are perfect and detecting animals during the subclinical period of infection is particularly challenging. Thus, there is a need for research into the sensitivity and specificity of tests that could detect infected animals before showing clinical signs. Availability of such test could enhance current control programs at both farm and country level. Some alternative testing methods are explained below.

#### 1.6.7.1 Blood PCR-phage

This laboratory technique tests a blood sample of cattle with a PCR. The blood sample needs to be processed using peptide-mediated magnetic separation followed by bacteriophage amplification before PCR is performed. This test allows detection 10 MAP cells per millilitre of blood and it works based on the principle that MAP infected animals become bacteriaemic after infection, thus MAP cells can be found in blood of MAP infected animals. Work have been done in clinical (Swift *et al.*, 2013) and subclinical animals (Swift *et al.*, 2016) and results show that it can detect infected animals effectively, although the test performs better in animals at the clinical stage. Its reported specificity and sensitivity was 100% and 35% respectively compared to faecal culture and/or milk serology result (O'Brien *et al.*, 2018). Further work and refinement of the blood process could increase sensitivity of this test.

#### 1.6.7.2 Biomarkers

*Selenium Glutathione Peroxidase (GSHPx)*

In a study performed in cattle and humans, the levels of GSHPx were compared between MAP infectious status groups. The hypothesis was that MAP infection (in both human and cattle populations) would cause an oxidative stress that would result in an increased GSHPx activity. Results showed that individuals with a positive blood MAP PCR result had significantly higher GSHPx concentrations than MAP negative subjects (Qasem *et al.*, 2016). Overlapping of results between infected and non-infected subjects occurred, so no threshold values were proposed to diagnose or exclude MAP infection status of the subjects studied. In

addition, no reference was made to diet implications that could have influenced the GSHPx blood levels in cattle. Despite its current limitations, more work could be performed in order to minimize confounding factors that could occur in cattle and compare this test with other tests (i.e. culture, PCR).

### Cholesterol

Levels of cholesterol were monitored after MAP exposure in infected animals. Results showed that cholesterol levels in blood at nine weeks post MAP inoculation increased significantly when compared to the cholesterol levels prior to MAP infection, but this cholesterol rise was temporary. Unfortunately, cholesterol levels at 13 weeks post infection declined and no differences between groups were seen (Johansen *et al.*, 2018). As a result, application of this biomarker as an indicator of MAP infection status is still of limited use. Although, this paper shows the interest of the scientific community in order to find biomarkers that could improve the diagnostic tools that are currently available.

**Table 1-1 Summary of the existing laboratory tests for Johne's disease.**

| Test | Se (%) | Sp (%) | Strengths | Weaknesses | Cost Lab | Comments |
|---|---|---|---|---|---|---|
| Culture | 33-49[a,b] | 100[a,b] | Gold standard. Detects viable MAP. High sensitivity | 42 days for the result. Can detect pass-through MAP | £44.00 | Can miss intermittent shedders |
| Clinical signs + culture | 70[b] | 100[b] | Rules out other wasting conditions. In increases the sensitivity of the culture result. | Needs time, experience and costs of veterinary services | £50 + £44 | Targets animals with a higher probability to suffer from Johne's disease. |
| Blood Serology | 15-40[a,b] | 99[a,b] | Fast. Very few false positives. | Sensitivity is low. Identifies positive animals when they are already infectious | £4.80 | Best performed in animals with clinical disease. |
| Clinical signs + blood serology | 83-95[b,c] | 99[b,c] | Rules out other wasting conditions. Increases Se and Sp from blood ELISA | Needs time, experience and costs of veterinary services | £50 + £4.80 | Milk production and/or history of diseases can help identify potential MAP-infected animals |
| Milk serology | 29-61[d] | 83-99[d] | Convenience of sampling | Lower se. and sp. compared to blood ELISA | £5.00 | Part of the routine milk quality control |
| PCR | 20-91[e] | 99[e] | Prompt results. | Can miss low shedders. Possibility of gastrointestinal pass through leads to false positives. | £29.50 | Can miss intermittent shedders |
| IFN-$\gamma$ | 50–85[f] | 80-95[f] | Allows identification of infected animals at an early stage. | Instability of the sample. Not available in most diagnostic laboratories. Technique still improving. | Not known | Promising, but still not available in the UK. Infected heifer calves could be discarded from breeding at an early stage. |
| Johnin test | NR | 57-100[g] | Cheap and fast. Detects cellular immunity. Potentially useful in subclinical cases. | Not available in the UK. Test performance varies a lot depending on Johnin PPD batch. | Not known | Can interfere with tuberculin test and Johne's serology |

| | Se | Sp | | | | |
|---|---|---|---|---|---|---|
| **Gross PM** | NR | NR | Becomes relevant in animals without any other test results. | Not confirmative. | £250 | Only recommended if clinical signs are present and animal not fit for human consumption. |
| **Histopa-thology** | 8-100[g,h] | 95[g,h] | Confirmative. It can identify infected animals with mild clinical signs. | If negative it is not exclusive. | + £47 | Result turnaround will vary between laboratories. |
| **Tissue PCR or culture** | NR | NR | Alternative to HE/ZN. Can be done after PM or at slaughterhouse. | Needs a good knowledge of intestinal anatomy to be performed correctly. | + £29.50 | Can be done as monitoring at slaughterhouse. |
| **Clinical signs alone** | NR | NR | Immediate results | Never confirmative. Non-specific. | £50 | Can be used in conjunction with other tests to target animals most likely to be positive. |

(Se) Sensitivity; (Sp) Specificity; (NR) Not reported. References: a - (Whitlock *et al.*, 2000); b - (Nielsen and Toft, 2008) c - Weber *et al.*, 2009); d - (Nielsen and Toft, 2008); e - (Leite *et al.*, 2013); f - (Huda, Jungersen and Lind, 2004); g - (Kalis *et al.*, 2015); h - (Martinson *et al.*, 2008), i - (Huda and Jensen, 2003)

# 1.7 Epidemiology of MAP

Johne's disease is prevalent in domestic ruminants worldwide and has a significant impact on the global economy (Sweeney, 1996). It is a disease included in the OIE-disease list and a notifiable disease in Scandinavian countries (Ulvund, 2012). MAP has also been isolated from other domestic animals, wildlife (Greig *et al.*, 1999; Beard *et al.*, 2001) and even humans (Kuenstner *et al.*, 2017). Despite the pathogenic impact on some animals not being proven as yet, the worldwide and broad host range make MAP a globally important pathogen.

## 1.7.1 Prevalence

### 1.7.1.1 Worldwide prevalence

Prevalence data of Johne's disease in different countries around the world are reported to be unavailable or inaccurate (Nielsen and Toft, 2009). However, the available data suggest that Johne's disease prevalence is high in the majority of the studied countries (Nielsen and Toft, 2009; Geraghty *et al.*, 2014). The Johne's disease distribution map of the OIE (OIE, 2017) (Figure 1-4) highlights examples of unavailable data (i.e. Turkey) or expected inaccurate data (i.e. eastern Europe, south-east Asia or South American countries). In this map the worldwide distribution of the disease is evident, especially within the most developed countries and countries with a high cattle population density.
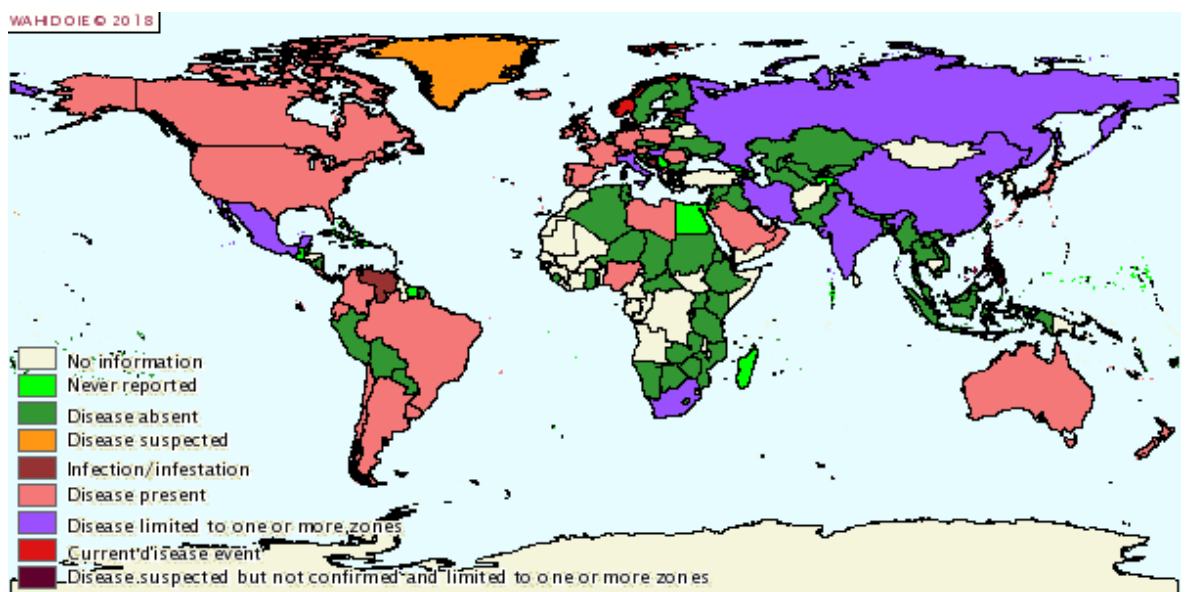


**Figure 1–9 Johne's disease distribution map in 2017, 6-month period, Jan-Jun; www.oie.int (2018)**

It is worth pointing out that most of the available data on Johne's disease prevalence concerns dairy cows and data for beef and sheep units is limited (Caldow and Gunn, 2009). Although, with the current data, countries can be divided regarding individual herd-level prevalence into:

- Low prevalence (<15%): Austria, Norway and Sweden (Caldow and Gunn, 2009; Nielsen and Toft, 2009)

- High prevalence (>15%): USA, UK, Denmark, Belgium and Caribbean countries (Nielsen and Toft, 2009; Fernández-Silva, Correa-Valencia and Ramírez, 2014)

### 1.7.1.2 United Kingdom and Ireland prevalence

Various estimates have been made regarding the prevalence of Johne's in the UK. In a study reported in 2006, 136 dairy herds were selected and 13,691 animals over three years old were tested over a period of six months using blood serology and culture on faecal samples. Results indicated that 34.7% of herds were affected by Johne's (Department for Environment Food and Rural Affairs, 2009). National milk records (NMR) performed an internal analysis using over 900 30-cow screens using milk serology from UK dairy herds performed over a 6-month period in 2011. One or more positive results were obtained in 68.9% of the herd screens having a within herd prevalence <6% (NMR 2011-unpublished data). There are few data available on the prevalence in beef herds in UK (Flook, 2015). Although, a study in Orkney showed a 66% herd-level prevalence in Orkney (Norquay, 2014)

In Ireland, prevalence was studied by Good et al. (2009). In this study they performed blood serology on 20,322 animals from 639 beef and dairy herds. Herd-level prevalence was 31.5% for dairy and of 17.9% for beef. Within herd prevalence was 19.6% for dairy herds; and 6.3% for beef herds.

Thus, available data suggest that Johne's disease in the UK and Ireland is well-established and infects a considerable number of farms and, therefore is of significant interest to both farmers and veterinarians.

## 1.7.2 Host range and wildlife role in MAP epidemiology

Apart from cattle and sheep, MAP also infects other ruminants such as goats and deer. MAP has also been reported in llamas and alpacas, normally in cases where there is contact with other farm animal ruminants (Fernández-Silva *et al.*, 2014). MAP has also been detected by faecal culture (without causing any disease) in non-ruminant species such as carnivorous or omnivorous predators (weasels, fox, stoat, crows, coyotes, raccoons) (Beard *et al.*, 2001) and prey animals (mice, rabbits, vole, rat, sparrows) (Beard *et al.*, 2001).

Disease is rare in these species except for some cases in rabbits, which seem to be the greatest potential wildlife vector in the spread of MAP in the UK. In Scotland, a study revealed that 67% of captured rabbits (*Oryctolagus cuniculus*) were infected with M. *paratuberculosis*. Infected animals were identified by a positive faecal culture result, or by detection of acid-fast bacteria on unspecified tissue on histopathology (Greig *et al.*, 1999). Association between wild rabbits and presence of historic Johne's disease on the farms was found in Scotland by Greig *et al.* (1999) and Daniels *et al.* (2001). Despite rabbits having histopathological lesions they were in good body condition, which suggests that MAP does not cause any clinical signs in this species. A study by Daniels *et al.*, (2001) also showed that cattle do not avoid grazing fields with rabbit faeces and ingestion of rabbit faeces occurred. Another study performed in Minnesota identified rabbits (*Sylvilagus floridanus*) and deer as a risk factor for dairy farms (Raizman *et al.*, 2005). Rabbits and deer positive for MAP on faecal culture were found in a higher proportion in farms with a positive pooled animal or farm environmental MAP culture sample. Although, in Minnesota the percentage of rabbits infected with MAP (identified by faecal culture) was 2%, a much lower number than the percentage obtained in Scotland (67%) by Greig *et al.* (1999). Prevalence difference in these two rabbit populations can be due to a true prevalence difference or the use of histopathology in the Scottish study, which increased the percentage of infected rabbits identified.

In conclusion, the role of wildlife in the spread of MAP has been shown to be relevant. Infected wildlife can serve as a vehicle to transmit bacteria between herds and has the potential to introduce MAP to non-infected herds. This is especially important in cases where young heifers (more susceptible to MAP

infection) are turned out to grass and complicates control measures that only rely on identifying infected bovine animals.

# 1.8 Economic impact

Annual losses due to paratuberculosis in the UK have been estimated at £9.8 million (range £4.2 to 15.4 million) for the dairy herd (based on a milk yield depression of 10%) and £3.1 million (range £1.33 to 4.88 million) for the beef herd (Caldow and Gunn, 2009).

## 1.8.1 Milk yield

Research into the economic impact of Johne's disease on dairy farms has been shown that MAP infection hampers production and profitability even prior to clinical disease (Johnson-Ifearulundu *et al.*, 2000). The most important economic loss is linked to decreased milk production, which is reduced by 4% to 20% on the serology positive cows compared to the negative herd mates (Gonda *et al.*, 2007; Richardson and More, 2009; Lombard, 2011). In more detail, Gonda et al. (2007) found that MAP-infected cows produced 303.9 kg less milk/lactation, 11.46 kg less fat/lactation, and 9.49 kg less protein/lactation when compared to non MAP-infected animals. Other studies however found no significant differences in production between animals with positive/negative Johne's serology results (Hoogendam *et al.*, 2009; Kennedy *et al.*, 2016). It may be that the differences reported in milk yield relate to what clinical stage of the disease the positive animals are at during the study period.

## 1.8.2 Further production impact

It has been reported paratuberculosis has also impact on other aspects of cow performance. Results suggested that serology positive cows were on average open for 28-days longer than seronegative cows (Johnson-Ifearulundu *et al.*, 2000). Other research showed that calving interval, strictly correlated to the number of open days did not seem affected by serology result (Hoogendam, Richardson and Mee, 2009).

MAP-infected cows have been found to be twice as likely to be culled for productivity reasons compared to non MAP-infected cows (Lombard, 2011). Premature culling associated costs are: increased replacement stock, loss of productive animals and genetic potential. Moreover, slaughter value of a clinically infected cow has been estimated to be 20–30% less than a non-infected

cow (Lombard, 2011; Radia *et al.*, 2013). On average these cows weigh 30 to 54 Kg less than their herd mates (Lombard, 2011). When cows suffering from Johne's disease reach an advanced stage, carcases with significant clinical signs such as emaciation and oedema may not be fit for consumption and so represent a complete loss of income to the famer (Lombard, 2011).

Regarding the impact of Johne's disease as a trigger for other conditions, a study estimated serology positive dairy cows were five times more likely to become lame and twice as likely to develop mastitis (Villarino and Jordan, 2005).

## 1.8.3 Beef herds

Less research has been carried out in beef herds regarding economic losses. Although existing data have proven that infected beef herds have also an impaired performance. Bhattarai et al. (2013) showed that cows with a high percentage of positivity on serology test produced calves that weighed 21.5Kg less at weaning than the mean of test negative animals. In addition, cows classed as moderate to high shedders by CFU on culture, produced calves with average weaning weight 58.5Kg less than mean of culture negative animals (Bhattarai *et al.*, 2013).

All these data illustrate how Johne's disease has an effect on different aspects of cattle production and health. Although, as different studies provide inconsistent data, it is still difficult to establish accurately which impact does Johne's disease have in an infected farm.

# 1.9 MAP control programs

Control programs have been established in different countries all over the globe. Different aims and strategies are set in each one. Testing approach, culling policy and results of some programs are described below to show the differences between them (Table 1-2).

## 1.9.1 Goal of the programs

National programs can vary depending on the end goal. While the Danish programme is targeted at reducing the prevalence of Johne's disease, the Dutch programme aims to reduce the amount of MAP in milk. In the Netherlands, individual regular milk serology is performed and the milk industry rejects milk from farms where an animal that tested positive has not been culled. This policy is thought to lower MAP burden to $10^3$/L, which regarding effectiveness of pasteurisation, would be low enough to eliminate MAP from retail milk (Geraghty *et al.*, 2014).

In Australia, the National Bovine Johne's Disease Strategic Plan goals include: a) reduction of MAP contamination of farms and farm products; b) protection of non-infected herds and regions; and c) reduction of any social, economic and trade impacts of MAP (Geraghty *et al.*, 2014). This program has shown that is possible to maintain disease free areas, with stringent controls on stock movement.

## 1.9.2 Culling policy

A particular example of a Johne's disease program is Japan, where paratuberculosis has been a notifiable disease since 1971. All cattle detected positive on faecal culture are culled and their farms are monitored until repeatedly test-negative. However, the prevalence of detected MAP cases has increased in the past 30 years irrespective of the fact that the disease has been notifiable, and there are no indications that the prevalence will decrease (Nielsen, 2009).

In Sweden, where the prevalence is very low, a rigorous culling programme of animals that test positive on culture and even culling the whole herd aims to

eradicate the disease (Caldow and Gunn, 2009). This is the only program in Europe where culling is mandatory, the rest of the European programs try to control Johne's disease by good husbandry practices and early detection of infected animals (Geraghty *et al.*, 2014).

**Table 1-2 Summary of the Johne's disease control programs in Australia, Canada, USA, Denmark, Netherlands and UK**

| Country and year programme started | Herd prevalence | % Herds involved | Unified Program | Compulsory / Voluntary and culling policy | Testing |
|---|---|---|---|---|---|
| Australia 1996 | Endemic in South East dairy and beef Rare or absent elsewhere | 95% | YES | Voluntary, but Johne's is notifiable and movement restrictions apply if farm is infected | All herds >300 cattle test all cows > 2 years old using blood serology Biennial test. Culture is used to follow up positive results. |
| Canada 2006 | Dairy (9.8–43.1%) Beef (7.9%) | 38-70% | NO | Voluntary. Positive cows must be culled within 90 days of testing and do not enter the food chain | All herds >100 cattle test all cows > 2 years for milk/blood serology Culture is used for farm environmental samples or pooled faecal samples (1/10) |
| USA 1998 | Dairy (68%) Beef (7.9%) | 9% 0.03% | NO | Voluntary. Positive cows cannot be moved interstate. | All cattle tested >36m males and >24m females using milk/blood serology. Definitive tests are faecal culture or PCR, tissue culture, PCR or histology |

| | | | | | |
|---|---|---|---|---|---|
| Denmark<br><br>1999 | Dairy (80–86%)<br><br>Present in beef herd at a lower prevalence | 28% | YES | Voluntary. Milk from "diseased" cows cannot be sold | Serology is required in all herds and >75% of the herd needs to have a test done within past year. Milk serology is used in dairy herds. |
| Netherlands<br><br>1952 | Dairy (20–71%)<br><br>Beef: no available data | 97% | YES | Compulsory. Milk is only collected from clean herds at most recent screen. All positive animals and their last-born calves must be culled. | All animals >3y old are tested on milk/blood serology.<br><br>Positives are re-tested by PCR or culture.<br><br>Faecal culture or PCR can also be used in pools of 1/5 in animals older than 2y of age. |
| UK<br><br>1998 | Dairy (27.6–42.5%)<br><br>Beef: no available data | 16% in CHeCS program | NO | Voluntary | Serology used in >2y old quarterly on milk or annually on serum.<br><br>Faecal PCR or culture are used to confirm serology tests. |

Sourced from (Geraghty *et al.*, 2014)

### 1.9.3 Control of MAP in the UK

Johne's disease control activities in the UK are delivered via multiple, independent groups. Ten separate programmes are run by breed societies and laboratories, each of which is licensed by the Cattle Health Certification Standards (CHeCS, established in 1998), which operates to a common Technical Standard regarding herd certification and control guidelines. (Geraghty *et al.*, 2014).

CHeCS offers two voluntary programs which farmers can enrol in (Brigstocke, 2017):

1. Johne's Disease Risk-Level Certification Programme (beef and dairy): aims to assign a risk level of Johne's disease at a farm level in order to allow the marketing of cattle with a certified risk-level. Cattle over 2 years old are tested for antibody on blood on an annual basis.

   Herds are classified with a risk level from 5 (high risk) to risk level 1 (low risk).

   • Level 1: Herds must have had three consecutive clear herd tests at annual intervals.

   • Level 2: This applies to all herds that have had an initial, or two consecutive clear tests, but are yet to achieve level 1 status.

   • Level 3: Number of test positive animals does not exceed 3% of the herd eligible for testing in the Johne's programme at the most recent test.

   • Level 4: These herds have more than 3% of eligible animals identified as test positive animals at the most recent test.

   • Level 5: Herds that are not adhering to the mandatory requirements of the programme.

Any animal that tests positive for antibody to Johne's disease by blood or milk ELISA needs additional testing. If they are negative for infective organism by

culture or PCR they should still be considered as high risk and must not be sold for breeding. It is also advised that these animals should not be retained for breeding (Brigstocke, 2017). These guidelines are based on the high specificity of the serology and the intermittent shedding of some animals, which can result in a false negative. Despite this, these animals do not affect the herd risk level. Additionally to herd testing, CHeCS program enforces herd health measures, which tackle the risk factor for introduction and spread of Johne's disease on farm (Brigstocke, 2017)

2. Johne's Disease Risk-Level Reduction Programme (dairy): to participate in this program you need to be enrolled in the program above. This program aims to reduce Johne's disease prevalence within the herd. The final goal is to achieve freedom from the disease.

   In this program individual cows have a risk level assigned depending on the results from the tests. All cows are divided into high, medium or low-risk groups. Thus, it advised that high risk cows are not bred.

   I. High Risk Cow: tested positive on two consecutive quarterly milk antibody tests or on one blood antibody test.

   II. Medium risk Cow: tested positive on one occasion in a quarterly milk antibody test during her current lactation.

   III. Low Risk Cow: more than two consecutive milk antibody tests or a single blood antibody test in the negative zone, including the most recent result, irrespective of previous testing results.

In addition to CHeCS accreditation, there is a national control program, Action Johne's, that aims to reduce the prevalence of the disease in British cattle (Action Johne's, 2018). The main parts of this program are:

- Training veterinarians and accredit them as Johne's veterinary advisors in order to assess farms and implement appropriate Johne's control plans on farm.

- Engaging farmers and reinforcing the importance of Johne's control.

- Encouraging milk buyers to have Johne's control plan's as a mandatory requirement for farmers that supply them with milk. This control plan has to be done with an accredited vet and a declaration has to be signed and it requires, a previous test in order to know the Johne's status of the farm. If the farmer does not sign to declare he will put the measures in place, he will not be able to sell any milk.

- The trained Johne's veterinarian and the farmer agree on applying one of the six control measures that are recommended to tackle Johne's disease on farm.

This control program is based on a very small investment by the government and it relies on farmers to pay for the testing and the veterinary visits. It also encourages farmers to enroll on the program as pressure from some milk buyers has made it compulsory. In comparison to other control programs, this one stresses the importance of farm practices rather than always relying on a "test and cull" policy. This means for herds with a high prevalence the disease can be managed realistically without having to cull a large proportion of the herd. Rather than identifying individuals, in this program, tests are used to indicate the status of Johne's infection on farm. The herd status is then used, in conjunction with advice from a trained vet, to implement the appropriate control measure. There are six strategies to control Johne's disease on farm:

1. Biosecurity, protect and monitor: for herds that have tested appropriately and have no evidence of the disease.

2. Improved farm management: this strategy aims to break the cycle of disease transmission from cow to calf. This option suits herds with low risk and low prevalence.

3. Improved farm management and strategic testing: this strategy uses individual cow testing to identify the cows at most risk of spreading Johne's disease while still reducing spread of disease from infected animals.

4. Improved farm management test and cull: immediate culling of the positives. This option would suit low prevalence herds.

5. Breed to terminal sire: all cows are served to a terminal sire and offspring are sold. All replacements have to be bought in. This strategy can suit farms with a high prevalence with little room to improve perinatal management.

6. Firebreak vaccination: vaccination efficacy is limited, it just reduces clinical signs and reduces MAP shedding. It can be used in high prevalence herds until another strategy is set in place.

These control strategies, which can be combined, aim to reduce the prevalence of Johne's disease in cattle from infected farms. This is a long-term goal that needs to be reviewed in a yearly basis by the accredited vet, which needs to monitor progress on Johne's disease control and make sure that the strategy implemented is working as expected.

## 1.9.4 Summary conclusions and aims of this study

In summary Johne's disease is costly and difficult to diagnose, with an as yet unknown zoonotic risk potential. In order to control the disease, identification of infected animals is vitally important. Currently available tools have a low sensitivity, which impairs the identification of infected animals, mainly at a sub-clinical stage. In order to make a correct clinical diagnosis, practitioners in the field have to understand the complex pathogenesis of the disease. Understanding of MAP shedding and antibody production are key in order to interpret diagnostic tests and make an accurate clinical diagnosis.

The present work aimed to describe the history, clinical presentation and Johne's disease test results from animals that were submitted to a teaching hospital with clinical signs of Johne's disease. Furthermore, it aimed to investigate the diagnostic test performance of a range of commercially available diagnostic tests in bovine cases with clinical signs suggestive of Johne's disease and compare them to the current gold standard test – faecal culture.

# Chapter 2    Materials and Methods

## 2.1 Case referral and hospital routine

The study was carried out in cattle presented to the farm animal hospital of the University of Glasgow, School of Veterinary Medicine (SVM) between January 2017 and June 2018. Cases for this study were referred to the teaching farm animal hospital of the University of Glasgow via a first opinion veterinarian. The cases are collected as part of the normal activities of the hospital, which aims to provide teaching cases (mainly cattle and sheep) for under and post graduate veterinary students. The system involves veterinarians working in general farm animal practice around Scotland. They identify animals from their practice that are subject to a disease or condition that are of interest that warrant further diagnostic investigation and /or that are no longer economic for the farmer to keep. Importantly, the veterinarians assess that the animal is fit for transportation to the hospital. When they identify a suitable case, first opinion veterinarians phone the hospital and the intern or resident on duty takes a history from the referring vet regarding the patient and herd. The intern or resident also contacts the farmer and records the history of the animal and details of the herd management (Appendix I).

Animals are transported to the hospital free of charge using stock workers trained in animal transportation. Farmers receive a £40 donation for each bovine case to help cover some of their costs. They also sign a consent form (Appendix II) to transfer ownership of the animal to the University of Glasgow and certify that they agree to data being used anonymously for research purposes. When the animal arrives, unless it needs emergency intervention, it is left a couple of hours to rest, drink and settle before it is examined. Cattle are usually individually penned with free access to water and haylage and supplementing of concentrates performed depending on individual animals' needs.

For the purpose of this study, animals that were referred to the hospital as part of this routine teaching referral process were used. For the most part, case investigation was part of normal clinical teaching activity, but some additional history questions and sampling was undertaken as detailed below.

## 2.2 Ethical approval

The use of clinical data from the animals included in the study for research purposes was approved by the Ethics & Welfare Committee of the School of Veterinary Medicine of the University of Glasgow, reference number 34a/18.

## 2.3 Animal history records

The history of both the individual animal and source herd from the referring vet and farmer was recorded in a paper format as part of the case file (see Appendix I). The following data were extracted from the history forms and then transferred into a spreadsheet (Microsoft Excel) for further analysis.

**Table 2-1 Information taken from the history forms**

| **Signalment** | Sex of the animal (male or female)[a] | Breed and purpose (breed name and beef/dairy) [a] | Age of the animal at referral (in months) [a] |
|---|---|---|---|
| **Herd and farm details** | Herd size (number of breeding cattle)[b] | Open or closed herd (the latter defined as not buying animals during last two years) [b] | Does the farm keep sheep? (yes/no) [b] |
| **Johne's history of the farm** | Did the farm have a confirmed Johne's case by a laboratory test in the last 5 years? (yes/no) [b] | Johne's control scheme member enrolment (yes/no) [b] | |

| **Individual clinical history** | One or multiple animals affected concurrently on the farm at time of referral. [b] | Onset of the clinical signs and date of last calving (difference between both dates expressed in months) [b] | Veterinarian's referral diagnosis (Johne's disease suspicion/ signs of weight loss and diarrhoea / other clinical signs) [a] |
|---|---|---|---|
| | | | |

a- Information recorded in a spreadsheet on the day of the animal's arrival as part of the routine recording system of the farm animal hospital. b- Information retrospectively transferred into a spreadsheet for the study purpose.

## 2.4 Clinical examination and inclusion criteria

A full clinical examination was carried out in the hospital facilities either at arrival or a few hours after the animal was settled. All animals that arrived in the hospital were assigned to Bachelor of Veterinary Medicine and Surgery (BVMS) final year production animal rotation students that performed and recorded results of a full clinical examination (Appendix III). A veterinary clinician (staff or post graduate student) also examined the animals and supervised students during their examination.

For the purpose of this study, bovines were included in the study if they met the two following case definition criteria:

- Animals with poor body condition score (BCS) lower than 2/5 OR a history of weight loss.
- Animals with the presence of diarrhoea (either persistent or intermittent).

Body condition score was assessed on the initial clinical exam and it was recorded in the individual case file (Appendix III). The history of weight loss was recalled during the history gathering of the animal prior to the animal's arrival

to the farm animal hospital (Appendix I). Records of the history were kept in the case file. Faecal consistency was monitored as part of the general assessment of the animal, thus if diarrhoea was noticed it was recorded on the daily clinical assessment sheet (Appendix VII). Hence, some animals were included in the study several days after arrival, when diarrhoea was noticed.

Study animals were assigned a hospital case number and were identified with a Johne's project sign displayed on the front of the pen.

## 2.5 Sampling

Cattle were sampled for diagnostic testing as part of the normal clinical work-up. For the purpose of this study, all animals had both a blood and faecal sample taken, with all samples labelled with the hospital case number. Samples were then subject to diagnostic testing as described in the following sections.

### 2.5.1 Routine Blood sampling

Heparin and Ethylenediaminetetraacetic acid (EDTA) blood samples were taken from all hospital cases and analysed for total protein (TP) and Packed Cell Volume (PCV). Further biochemistry and haematology tests were carried out at the discretion of the veterinarian on duty in the clinic. All cases that met the inclusion criteria also had a blood sample taken into a plain vacutainer tube. The plain tube was then cleaned, labelled and stored in a fridge awaiting to be sent to the laboratory for ELISA testing.

#### 2.5.1.1 Haematology and biochemistry

For all cases heparin and EDTA blood was also sent to the University of Glasgow Veterinary Diagnostic services laboratory, where further biochemistry and haematology analysis was carried out. For the purpose of this study, albumin and globulin blood levels (as part of the basic ruminant biochemistry profile) and PCV values (as part of haematology report) were used for further analysis.

Albumin measurement was done by the bromocresol green method using the chemistry analyser Dimension Xpand Plus (Siemens, Frimley, UK). Total protein was measured using the Biuret method with the same chemistry analyser

described before and Globulins were calculated by subtracting albumin from the total protein measurement.

All the results were sent by e-mail to the farm animal department (see Appendix IV) and recorded in the animal's case file.

## 2.5.2 Faecal sampling

A faecal sample was obtained during rectal examination which was part of the clinical examination. A non-sterile, airtight pot was filled with faeces, cleaned, labelled and stored in the fridge together with the blood sample and the corresponding filled laboratory form.

# 2.6 Diagnostic testing

The plain blood tube and faecal sample were sent by regular mail, meeting criteria for postage of biological material, to SRUC (Scotland's Rural College) Ayr laboratory and from there forwarded to the SRUC Edinburgh laboratory. The laboratory tests requested for each case were: Johne's serology, polymerase-chain reaction (PCR) and Culture (see Appendix V)

## 2.6.1 Serology

Blood samples from all study animals were subject to a Johne's serology test performed using serum in a clotted blood tube. This was an indirect absorbed ELISA for the detection of anti-Mycobacterium avium subsp. paratuberculosis (MAP) antibodies (ID Screen® Paratuberculosis, IDVet Genetics).

Results were reported back by e-mail within two weeks after the samples were sent (see Appendix VI). The result was expressed in qualitative manner (positive or negative) and in percentage of positivity in relation to a positive control.

## 2.6.2 Faecal PCR

Faecal samples from all study animals were subject to a Johne's disease PCR test, which targets MAP genome within the sample. This was a real-time PCR

(VetMAX MAP Screening Kit, Thermofisher/Life Technologies). This test specifically targets the gene ISMAP02, a specific gene of MAP.

Results were reported back by e-mail within two weeks after the samples were sent (see Appendix VI). The result was expressed in qualitative manner (positive or negative). The qualitative results were followed with a CT (Cycle threshold) values of the positive animals displayed in a spreadsheet (Microsoft Excel) that were sent especially by e-mail for this research project.

### 2.6.3 Faecal Culture

Faecal samples from all animals were subject to a culture process to look for the presence of growth of MAP organisms. Faecal material was cultured in a liquid Mycobacterium selective media (para-JEM broth media, Thermofisher/Life Technologies) for 42 days. The resulting broth was then analysed with the same PCR kit specified previously and confirmed MAP positive or negative.

Results were reported by e-mail six weeks after the samples were sent (See Appendix VI). The result was expressed in a qualitative manner (positive or negative). Subsequently, the CT values of the positive animals were reported in an additional spreadsheet that was sent to the researchers for the purpose of this study.

### 2.6.4 Case recording

All clinical pathology and Johne's disease test results were gathered in an individual case electronic folder for each case and results were entered into a spreadsheet (Microsoft Excel). This spreadsheet is described below (Section 2.9).

## 2.7 Case follow up

All animals were under the care of the veterinarians and students on duty in the hospital. They received daily clinical examinations, which included as a minimum: rectal temperature, respiratory rate and heart rate (see Appendix VII). During morning clinical rounds, students presented the case to their peers and the duty clinician as well as any updates or results. A case discussion was performed with the clinician and an action plan agreed each day.

## 2.7.1 Case outcome

When it was deemed that the animals were no longer suitable for teaching, clinical diagnosis was reached or, if it was anticipated welfare could be compromised, animals were euthanized by intravenous injection of pentobarbitone sodium 200 mg/ml at the hospital facilities. In cases where the animal deteriorated suddenly, euthanasia was performed regardless of the stage of the clinical investigation. All animals were then transported to the necropsy room, which was in very close proximity to the farm animal hospital.

# 2.8 Post-mortem examination

Post-mortem examination was performed by an experienced pathologist (five different pathologists were working on a rotational basis) and final year veterinary students. Each animal had a full gross post-mortem examination followed by a necropsy discussion between the farm animal department students, farm animal clinicians, pathology students and pathologists. In every case, a section of approximately 7cm around the Ileo-caecal valve (including a part of ileum and a part of caecum) and the whole ileo-caecal lymph node were collected. These samples were stored in a 10% formalin solution for at least one week before being processed for histology examination.

A report of gross pathology findings was sent by e-mail to the farm animal department (see Appendix VIII). In all reports the following items were reported:

**Table 2-2 Lesion scoring system used on the PM examination**

| | | | | |
|---|---|---|---|---|
| **Lesions consistent with Johne's disease (intestines and mesenteric lymph-nodes)** | Zero | One | Two | Three |
| | Absence of lesions | Mild lesions | Moderate lesions | Severe lesions |
| **Liver fluke lesions in the liver** | Zero | One | Two | Three |
| | Absence of lesions | Mild lesions | Moderate lesions | Severe lesions |
| **Live fluke presence** | Yes | No | | |

## 2.8.1 Histology preparation

One trained researcher cut the formalin fixed tissues with a scalpel. A thin slice was obtained from each sampled tissue. The resulting cuts had to fit in histology cassettes of 26mm x 30mm x 5mm dimensions. The lymph node and Ileo-caecal valve cuts were transferred into a pot with a 10% formalin solution and sent to the histopathology laboratory for slide preparation and haematoxylin eosin (H/E) and Ziehl Neelsen (ZN) staining.

A total of four slides were prepared for each case:
1. Ileo-caecal valve ZN stain
2. Ileo-caecal valve H/E stain
3. Lymph node ZN stain
4. Lymph node H/E stain

## 2.8.2 Histological examination

Histological examination of the ileo-caecal valve and proximal lymph node was carried out on the samples obtained from the PM. Every case had two ZN stained

slides, which were examined for 10 minutes each and with a maximum amplification of 400X. Results were recorded as positive or negative depending on the presence or absence of acid-fast bacteria (Figure 2-1). These are classically intracellular, bright magenta small rods that normally are found within macrophages. H/E slides were prepared for completeness, but they were not needed in any of the cases.
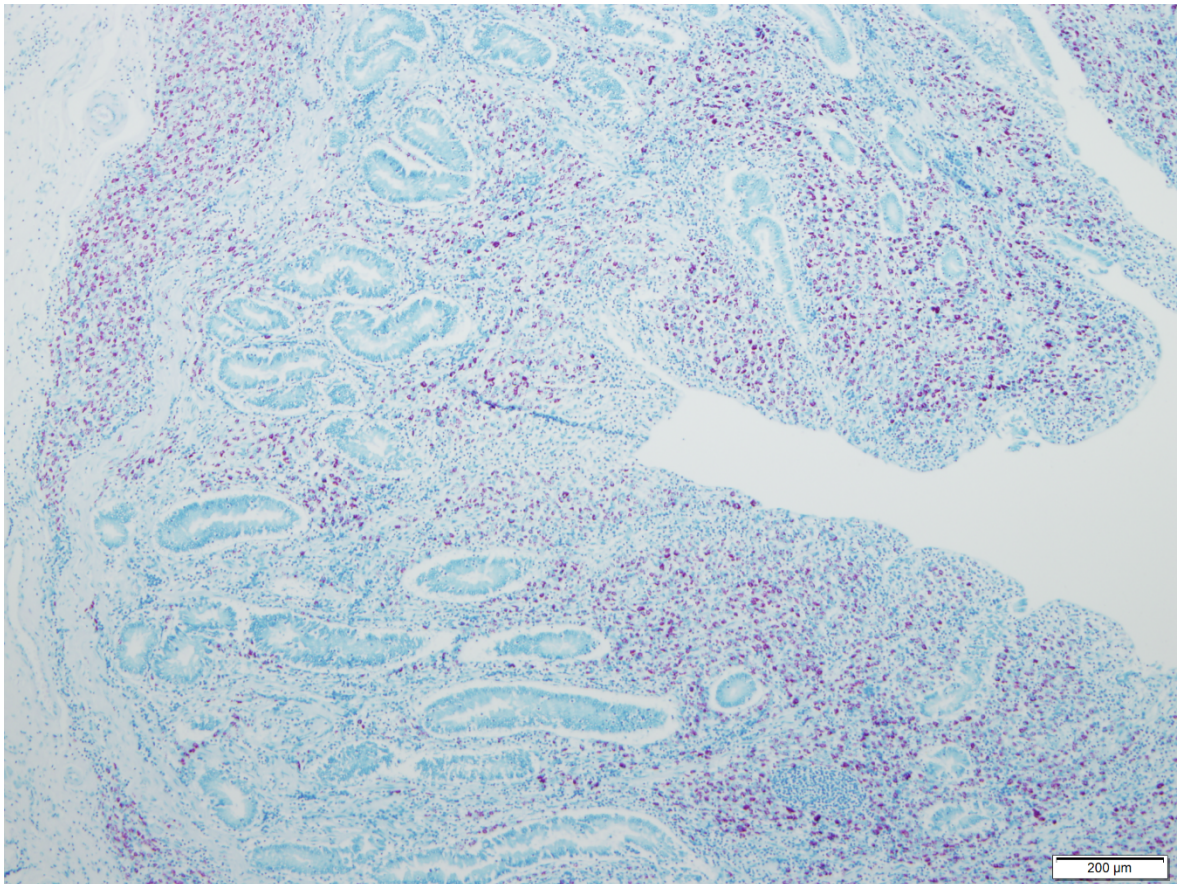


**Figure 2–1 Picture of a section of the ileo-caecal valve stained with a ZN stain, case 264054. A significant number of acid-fast bacteria appear stained purple-magenta colour within the intestinal parenchyma, which is stained in clear blue colour.**
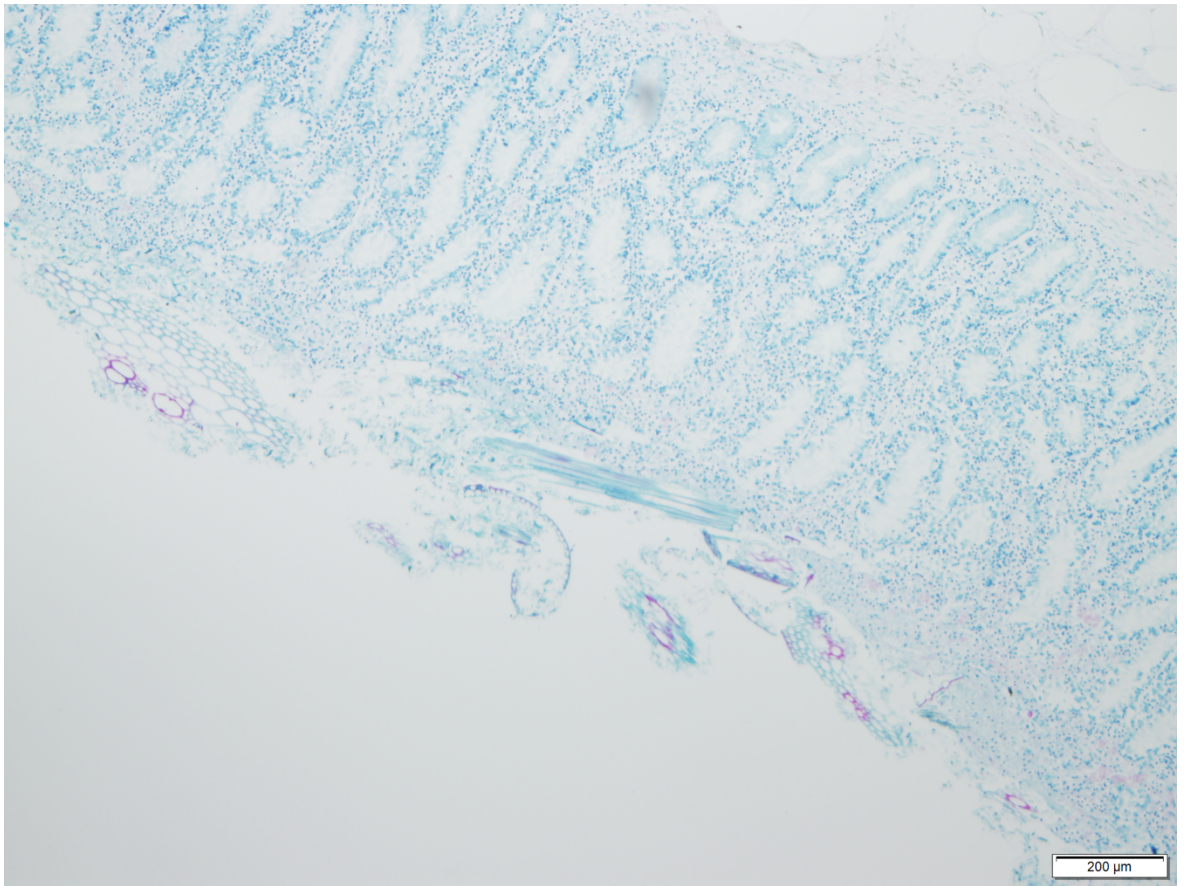
**Figure 2–2 Picture of a section proximal to the ileo-caecal valve stained with a ZN stain, case 244724. No acid-fast bacteria were present in the sections examined. However, plant material within the lumen appear stained in purple-magenta colour. The rest of the intestinal parenchyma is stained in clear blue colour.**

## 2.9 Data storage

All data were collected and stored in a spreadsheet (Microsoft Excel). Animal signalment, owner details, history from the farmer, history from the vet, referral diagnosis, clinical exam findings, clinical diagnosis in the farm animal hospital, SRUC Johne's test results, haematology and biochemistry results, PM findings and histology results were all collected in the definitive spreadsheet (see Appendix IX).

In the final spreadsheet the following fields were included:

**Table 2-3 Fields included in the final spreadsheet**

| Signalment | Origin of the animal | History of the farm |
|---|---|---|
| Case number | Name of the owner and farm | Herd size |
| Date in (xx/xx/xxxx) | Postcode | Open or closed |
| PM number | Phone number | Sheep co-grazing (yes/no) |
| Animal ear tag | Referring vet | Johne's status (positive/assumed negative) |
| SRUC reference number | Referring diagnosis | Control scheme enrolment (yes/no) |
| Breed | | Last TB test date (xx/xx/xxxx) |
| Sex (M/F) | | Contact with animals from other farms |
| Age of sampling (age in months) | | |
| **Clinical data** | **MAP laboratory results** | **PM results** |
| Clinical diagnosis | PCR result (+/-) | PM diagnosis |
| Clinical signs (consistent with Johne's /additional clinical signs) | PCR CT value | Liver fluke lesions grade (0-3) |

| Albumin results | Culture result (+/-) | Alive liver fluke (+/-) |
|---|---|---|
| Globulin results | Culture CT value | Alive rumen fluke (+/-) |
| PCV results (%) | Serology qualitative result (+/-) | Nematodes infestation (*Ostertagia* spp. or intestinal nematodes) |
| BCS results (1-5/5) | Serology quantitative result | Gross pathology consistent with Johne's disease (+/-) |
| | | Enlarged lymph nodes (+/-) |
| | | Presence of oedema (+/-) |
| | | Ileo-caecal valve histology ZN result (+/-) |
| | | Ileo-caecal lymph node histology ZN result (+/-) |

## 2.10 Data analysis

### 2.10.1    Descriptive data

Data on signalment, origin of the animals, history of the farm, clinical data, MAP laboratory results and PM results were summarised and presented in tables and histogram graphs.

## 2.10.2 Statistical analysis

Analyses of data were performed using Minitab®18. The different statistical tests used and data analysed are listed below. The significance cut-off value chosen was p = 0.05:

- Chi-square for association, using the Pearson p-value for the following data: herd size, biosecurity, sheep co-grazing, known positive farm, health scheme, previous positive test, number of affected animals, clinical signs onset, referral diagnosis, sex, purpose, clinical exam, albumin concentration, globulin concentration, serology, PCR, post-mortem, histopathology, parasite infestation status, culture and PCR CT values.

- Odds Ratio and 95% confidence level intervals for the following data: biosecurity, sheep co-grazing, known positive farm, health scheme, clinical signs onset, clinical exam, serology, PCR, post-mortem, histopathology, culture and PCR CT values.

- Mann-Whitney test on the following data: body condition score, albumin concentration, globulin concentration, culture and PCR CT values.

- Scatter-plot and fitted line regression: PCR CT values and serology positivity percentage correlation.

Formulas to assess sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were applied as follows:

| Test X | Culture | | |
|---|---|---|---|
| | Positive | Negative | Total |
| Positive | A (true positives) | B (false positives) | A+B |
| Negative | C (false negatives) | D (true negatives) | C+D |
| Total | A+C | B+D | A+B+C+D |

Sensitivity: A/A+C; Specificity: D/B+D; PPV: A/A+B; NPV: D/C+D

The author of the present study actively participated in the the following tasks: Ethical approval, inclusion decision (based on inclusion criteria), animal history recording, clinical examination, sampling of the animals, case follow-up, data storage, animal histology sampling, formalin-fixed tissue cuts, ZN slide interpretation and data analysis. The author did not perform the following tasks: haematology and biochemistry, Johne's laboratory diagnostic tests (serology, PCR, culture), post-mortem examinations, histology cut preparations.

# Chapter 3    Results

## 3.1 Description of the population studied

A total of 192 bovine cases were admitted to the farm animal teaching hospital for a range of different clinical presentations during the study period of the 6th of January 2017 and the 16th of June 2018. All cases were from Scotland and within a 221-mile radius of the SVM.

In total 59 animals met the inclusion criteria (see Chapter 2: Materials and methods) for the project. A total of 52 females and 7 males were sampled. Referrals were sent by 26 different vets from 15 different practices. Cattle came from 51 different farms from Scotland with ages ranging from 9 months to 15 years of age (median of 4 years 4 months of age). Fifteen different breeds were included in the study, Holstein (17), Jersey (5), Limousin (4) and Simmental (4) were the most represented breeds. In total, 25 dairy and 34 beef cattle met the inclusion criteria.

From the initial 59 cattle, 49 animals had all the Johne's laboratory tests performed (ELISA, PCR and culture) and 33 had all the antemortem tests plus post mortem and additional histology examination performed.

## 3.2 Case clinical history

From the 49 cases that had ELISA, PCR and culture results, three cases had history data missing giving a complete data set for case clinical history for 46 animals. For each of the history parameters presented, data are described in total and presented in relation to the culture result.

### 3.2.1 Herd size

The farms of origin were classified into three different groups according to the number of breeding cattle that they had: small farms (1-100 breeding cows), medium size farms (101-300 breeding cows) and large size farms (>301 breeding cows). Within the small farm group, seven animals tested positive and five were negative on culture. Within medium size farms, eight animals were positive and 12 animals were negative on culture. From the group of larger farms, seven

animals were positive and the other seven were negative on culture (Figure 3-1). The greater proportion of positive results by group was found in the smaller size farms, although the differences seen between groups were not significant (p=0.592).
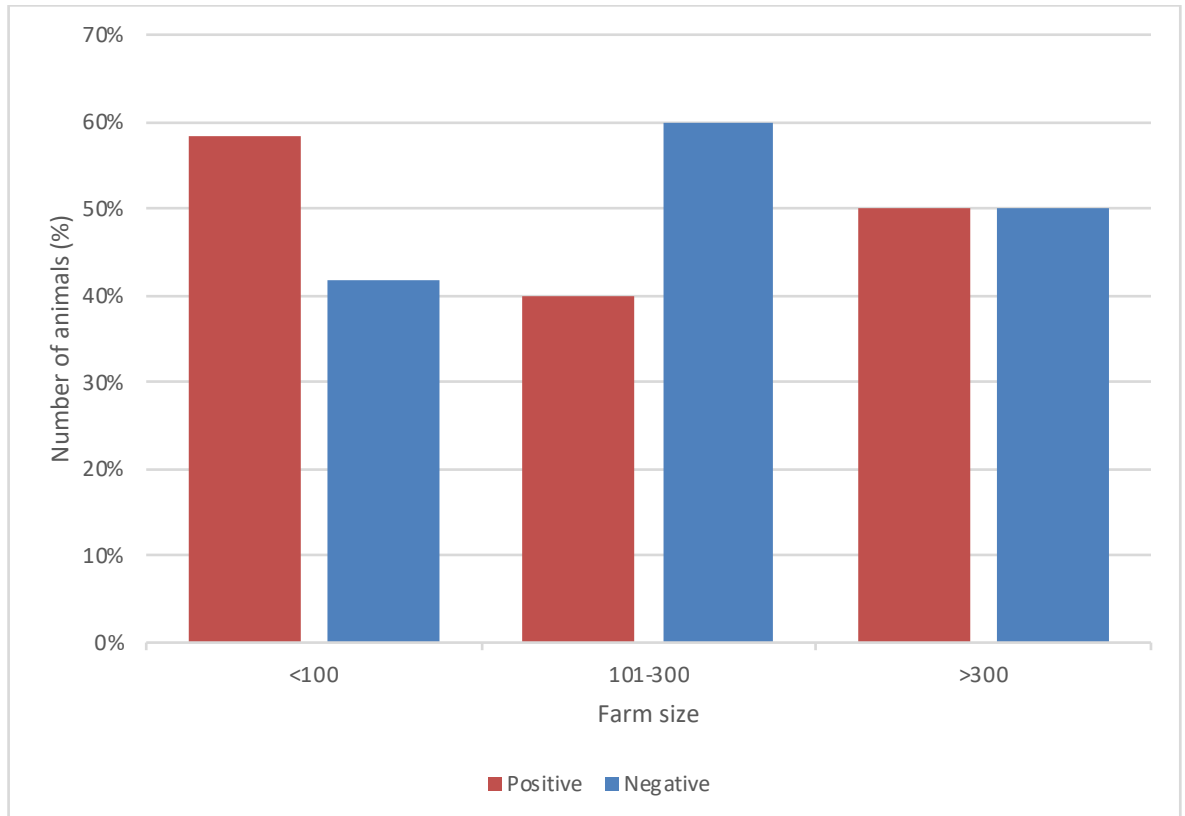


**Figure 3–1 Culture results shown by herd size of the farm of origin.**

## 3.2.2 Biosecurity status of the herd: open/closed herd

A total of 26 animals were submitted from herds described as 'open' (see Chapter 2 Materials and methods) by the owner. From these, 11 animals tested positive on culture and 15 animals were negative on the same test. From the twenty animals that arrived from closed herds (defined as not buying animals during last two years), 11 animals tested positive and nine animals tested negative on culture (Figure 3-2). Thus, the odds ratio (OR) of testing positive from an open herd compared to a closed herd was 0.60, with a 95% confidence interval (95% CI) of 0.185-1.943 (p=0.333), suggesting that the biosecurity status of the farm had no observed effect on the Johne's culture outcome.
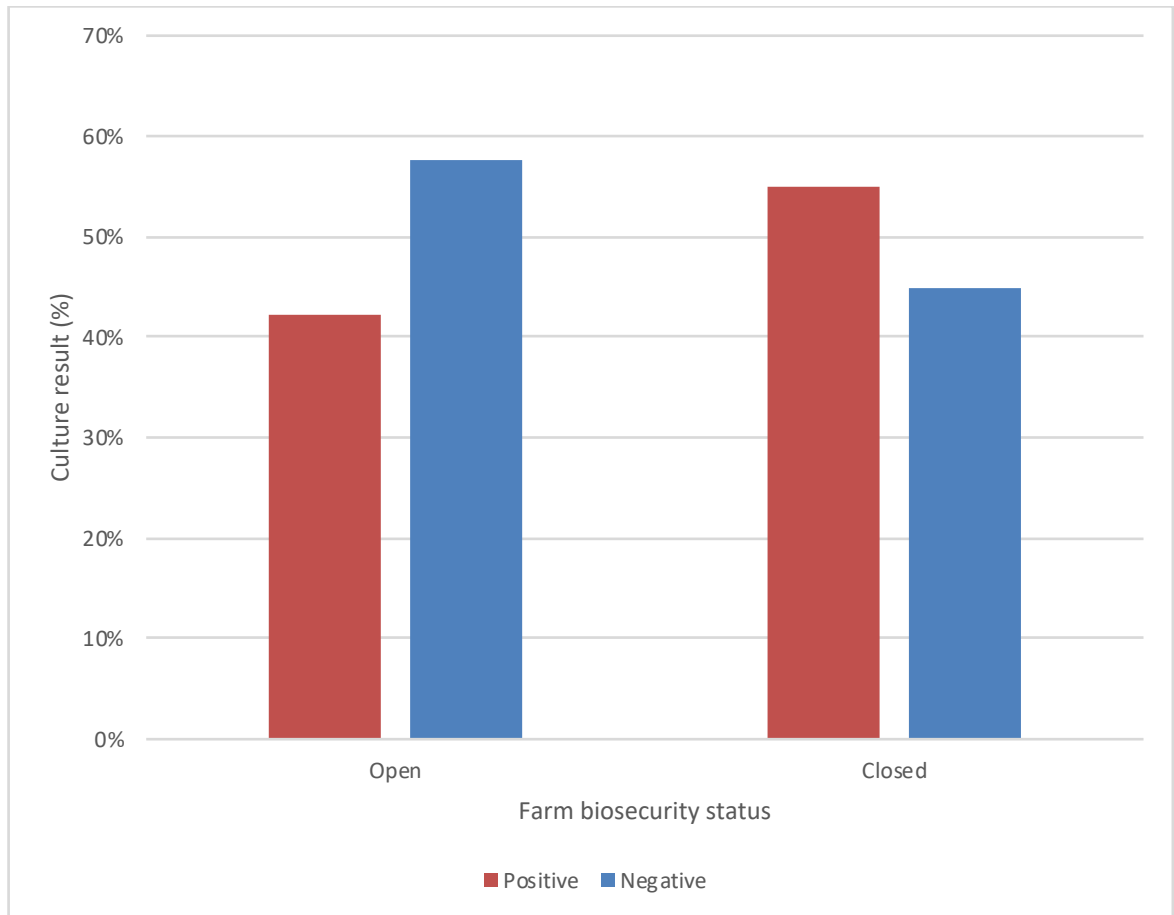
**Figure 3–2 Culture results shown by the biosecurity status (open/closed) on the farm of origin.**

## 3.2.3 Co-grazing sheep

A total of 19 animals were referred from farms that also had sheep. From those, 11 and eight animals tested positive and negative on culture respectively (Figure 3-3). The rest of the 27 animals came from farms with no sheep. From those, 11 and 16 animals tested positive and negative on culture respectively. The higher proportion of animals coming from co-grazing herds that were culture positive had an OR of 2.00 with a 95% CI= 0.608-6.581 with no observed differences between these two groups (p= 0.251). Thus, no relation between co-grazing and culture outcome could be concluded based on the animals sampled.
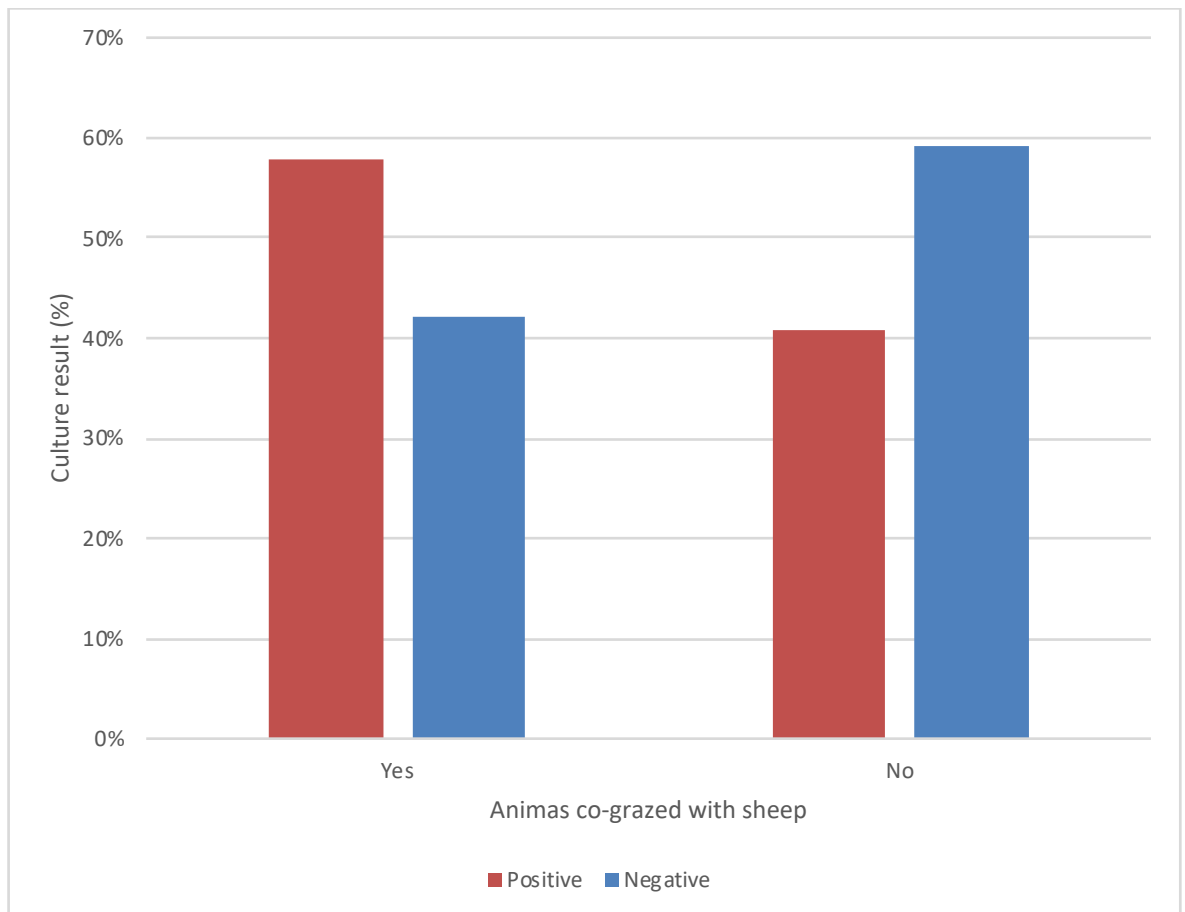
**Figure 3–3 Culture results shown by sheep co-grazing factor (yes/no) at the farm of origin.**

## 3.2.4 History of a positive test in the farm of origin

A total of 25 animals arrived from farms with a farmer reported history of a confirmed positive result in the past five years. From those, 15 were positive and 10 tested negative on culture (Figure 3-4). From the 21 animals that arrived from farms with no previous positive result, seven animals were positive on culture, being the first evidenced notice of Johne's disease on farm and fourteen animals tested negative. A higher proportion of positive animals was found in animals from known Johne's positive farms OR=3 (95% CI=0.8948 - 10.0576). Despite the tendency shown, these differences were not significant between groups (p=0.071).
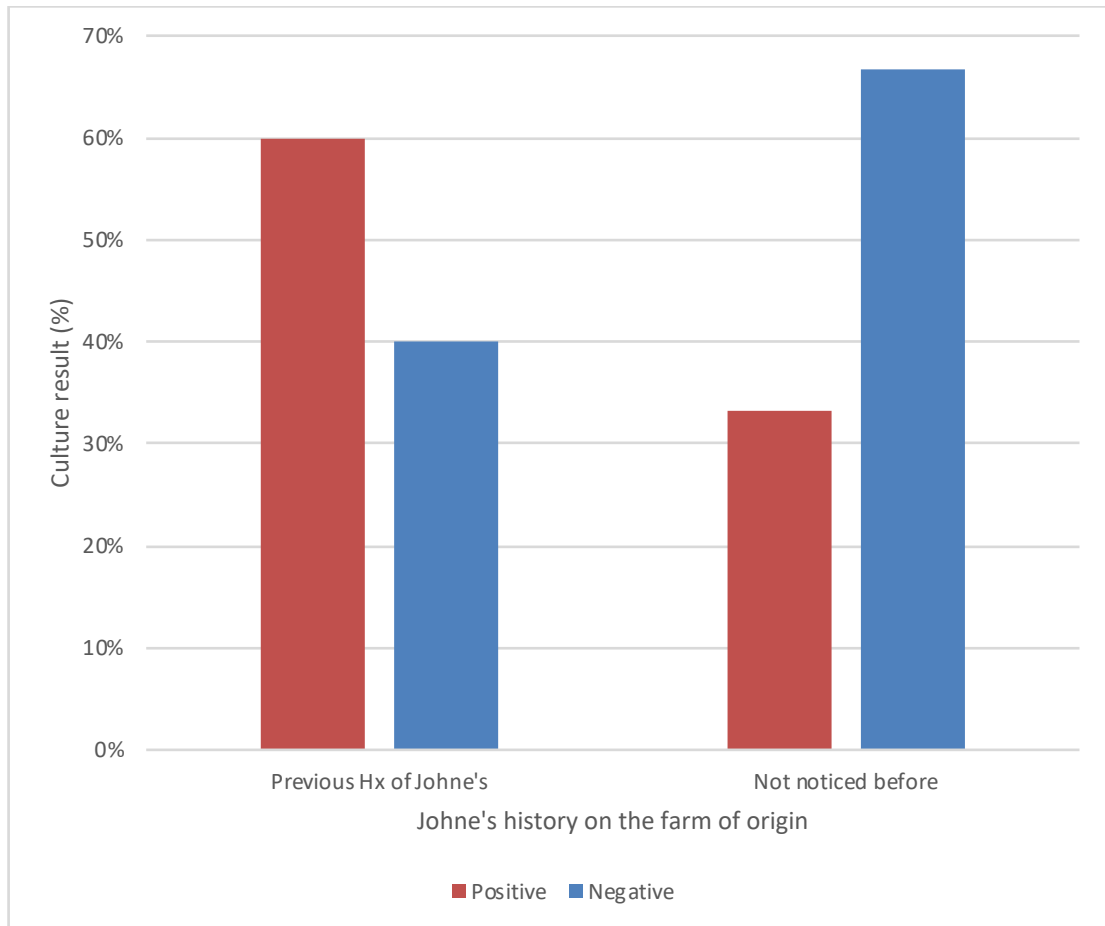
**Figure 3–4 Culture result shown by the previous evidence of Johne's disease on farm (Previous history of Johne's/ Not noticed before).**

## 3.2.5 Johne's health scheme membership

A total of 42 cases came from farms that were not a member of any Johne's control health schemes (Figure 3-5). From those, 20 tested positive and 22 tested negative on culture. Four animals were referred from farms that were a member of a Johne's control health schemes. From those, two cases were positive and the two others were negative on culture. Differences between groups observed in this case had an OR=1.10 (95% CI= 0.1414 – 8.5565) and were not significant (p=0.927).
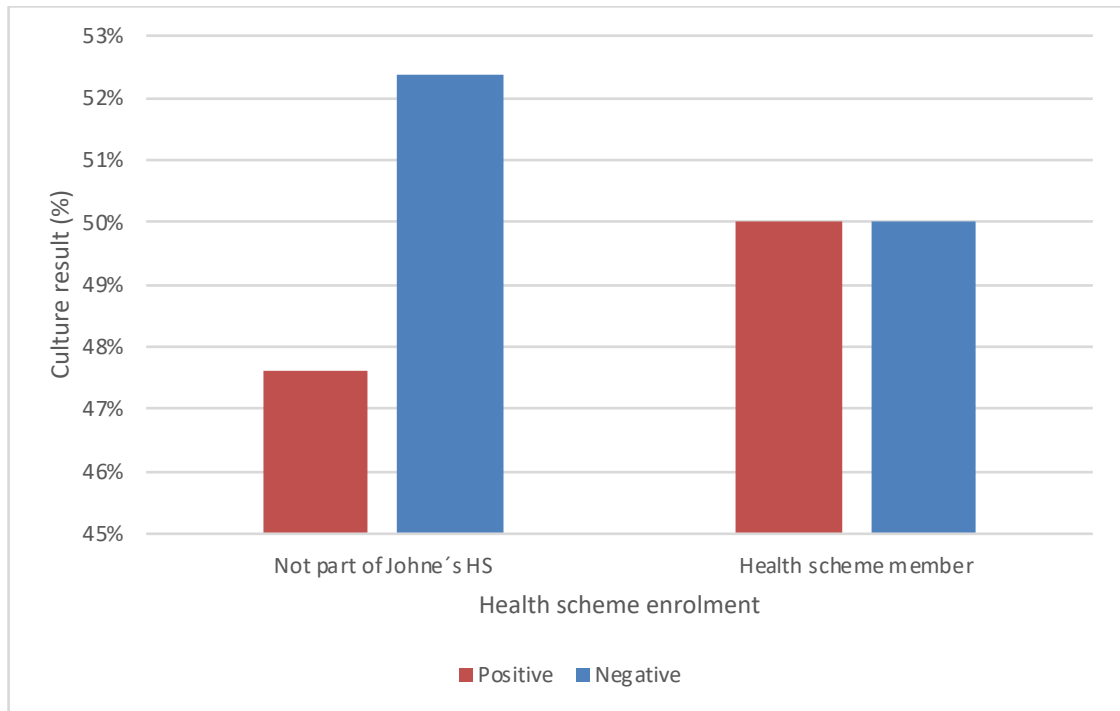
**Figure 3–5 Culture result shown by health scheme enrolment (not part of a Johne's control health scheme/health scheme member).**

## 3.2.6 Previous Johne's testing prior to admission

The majority of cattle (37/46) were referred without any previous Johne's test performed on the farm of origin (Figure 3-6). From the six animals that were tested positive on farm, all of them were confirmed positive on culture. From the three animals that tested negative on farm one of them was found to be positive (tested PCR negative on farm). This animal tested positive on Serology, PCR and Culture in the hospital. The test history on farm was significantly positively associated with the Johne's culture outcome (p=0.023).
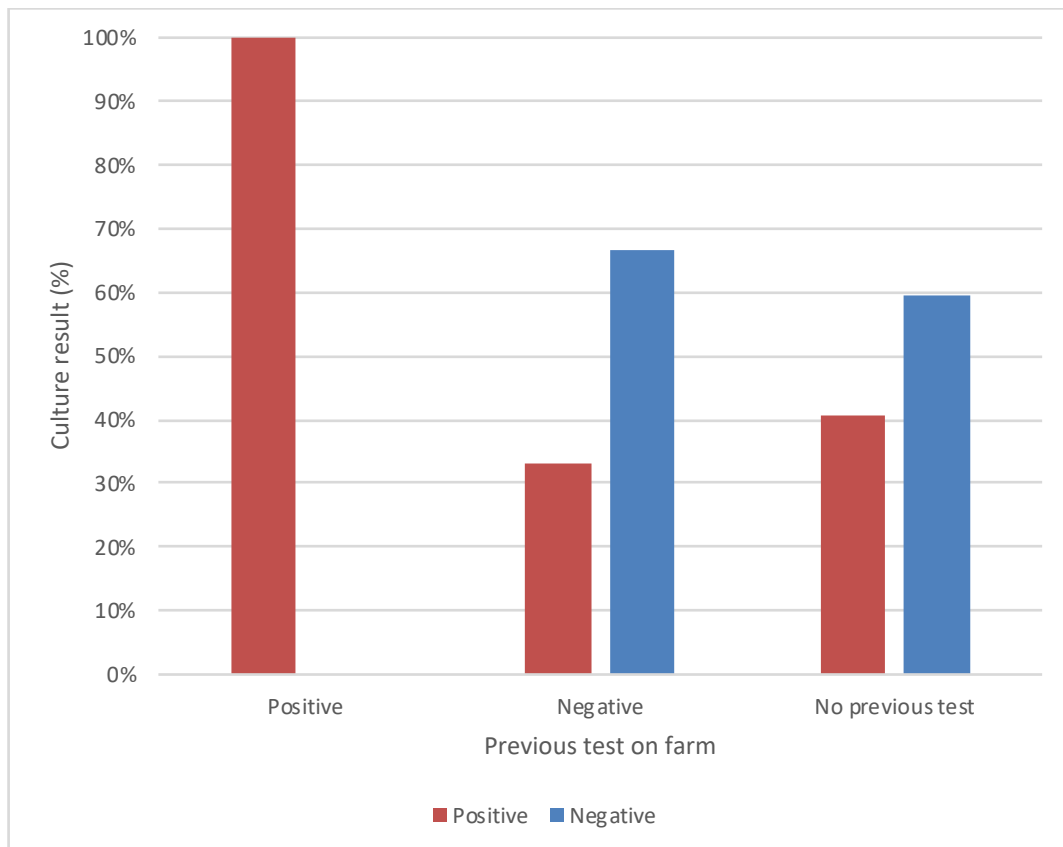
**Figure 3–6 Culture results shown by the test history on farm (positive/negative/not tested).**

## 3.2.7 Single or multiple animals affected?

A total of 11 animals were referred from farms with concurrently, more than one animal showing similar clinical signs. From those, eight animals tested positive and three other animals tested negative on culture (Figure 3-7). The 35 animals that were referred as a single animal problem were 14 culture positive and 21 culture negative animals. A higher proportion of positive results were found from animals referred from farms with multiple animals affected with an OR=4.00 (95% CI= 0.7655 – 17.7454). Despite the tendency shown, these differences were not significant between groups (p=0.058).
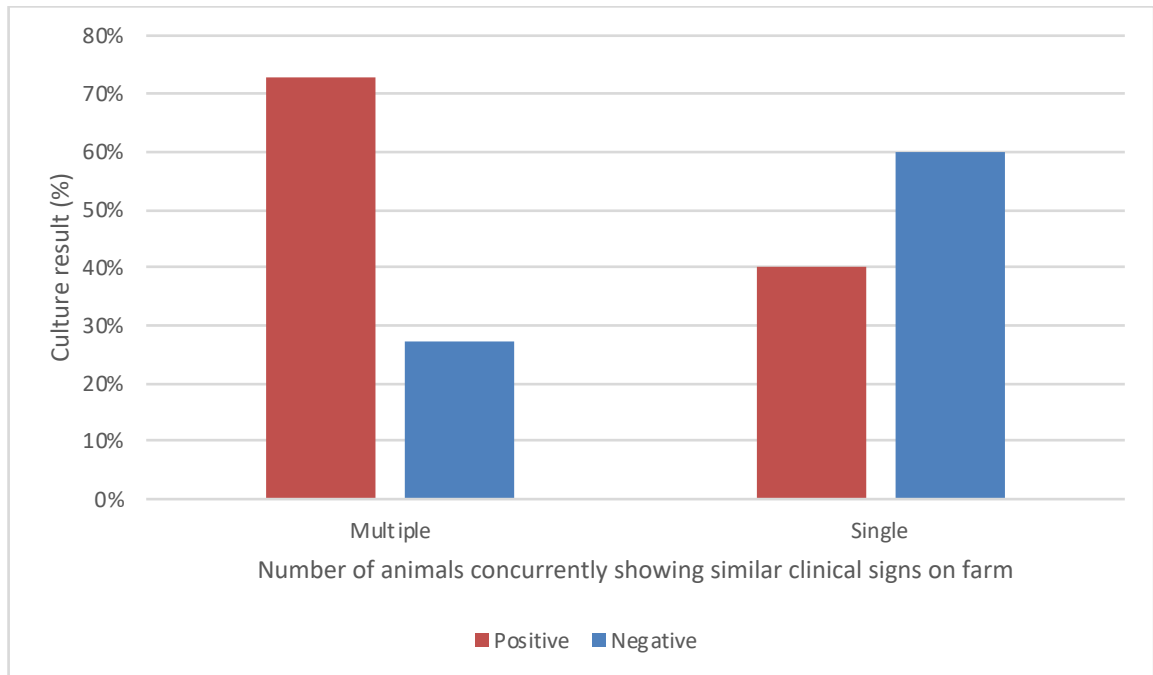
**Figure 3–7 Culture results shown by number of animals concurrently showing similar clinical signs (single/multiple) on the farm of origin.**

## 3.2.8 Onset of the clinical signs

A total of 35 female adult cows had calved. From those, 16/35 (46%) cases were referred with a history of the onset of the disease being within the first two months after calving. From those, six animals were culture positive and ten were culture negative. The 19 animals remaining had 12 and 7 positive and negative results on culture respectively (Figure 3-8). A slightly higher proportion of culture positive animals was found in animals with a history of becoming ill during two months post-calving with an OR= 0.35 (95% CI= 0.0884 – 1.3856). These differences were not significant between groups (p=0.373).
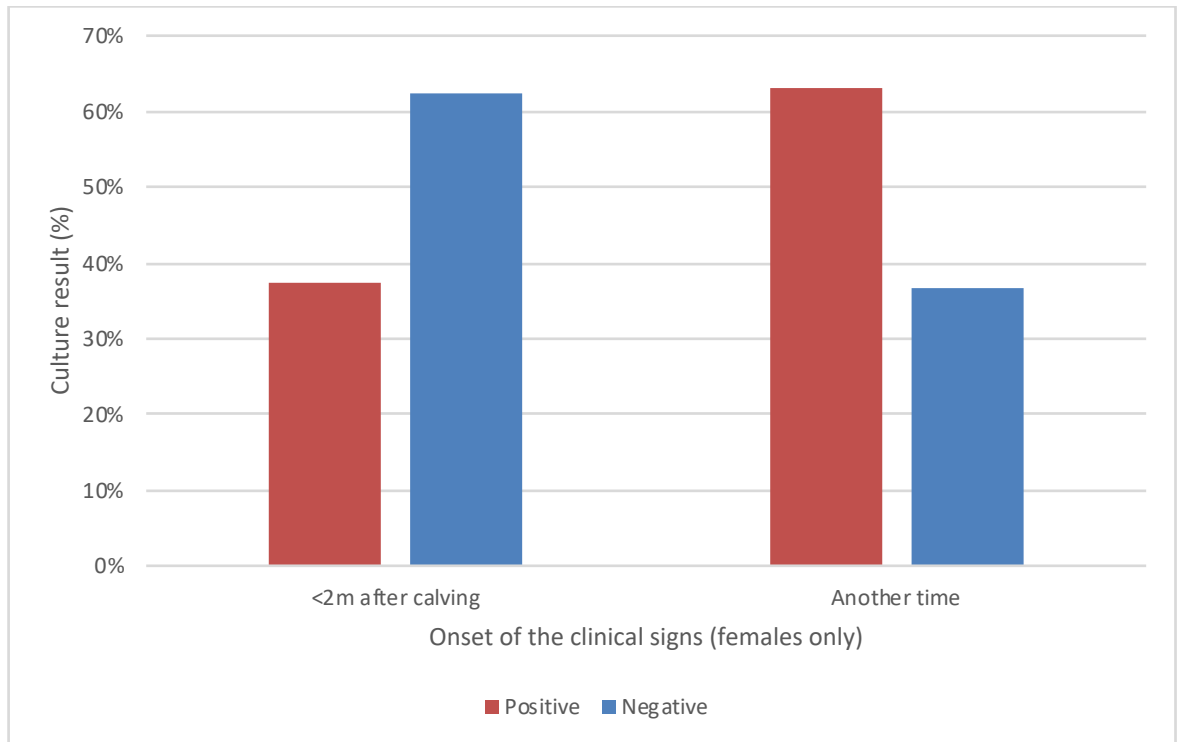
**Figure 3–8 Culture results shown by onset of the disease (within two months after calving/another time).**

## 3.2.9 Referral diagnosis

Information about the referral diagnosis was available for all the animals that met the inclusion criteria (49 animals). A total of 13 cases were referred with a suspicion of Johne's disease from the referring vet. From those 13 animals, twelve were confirmed by a positive culture result and one case was negative on culture (Figure 3-9). Another 13 cases were referred with a history of weight loss and/or diarrhoea (i.e. the referring veterinary surgeon did not suspect Johne's disease as the primary complaint). Five of them were positive and the other eight were negative on culture. From the other 22 cases referred for other reasons, but which also fitted the inclusion criteria for the study, six tested positive on culture and 17 were negative on culture. The difference in culture results between the three groups were significant (p=0.001).
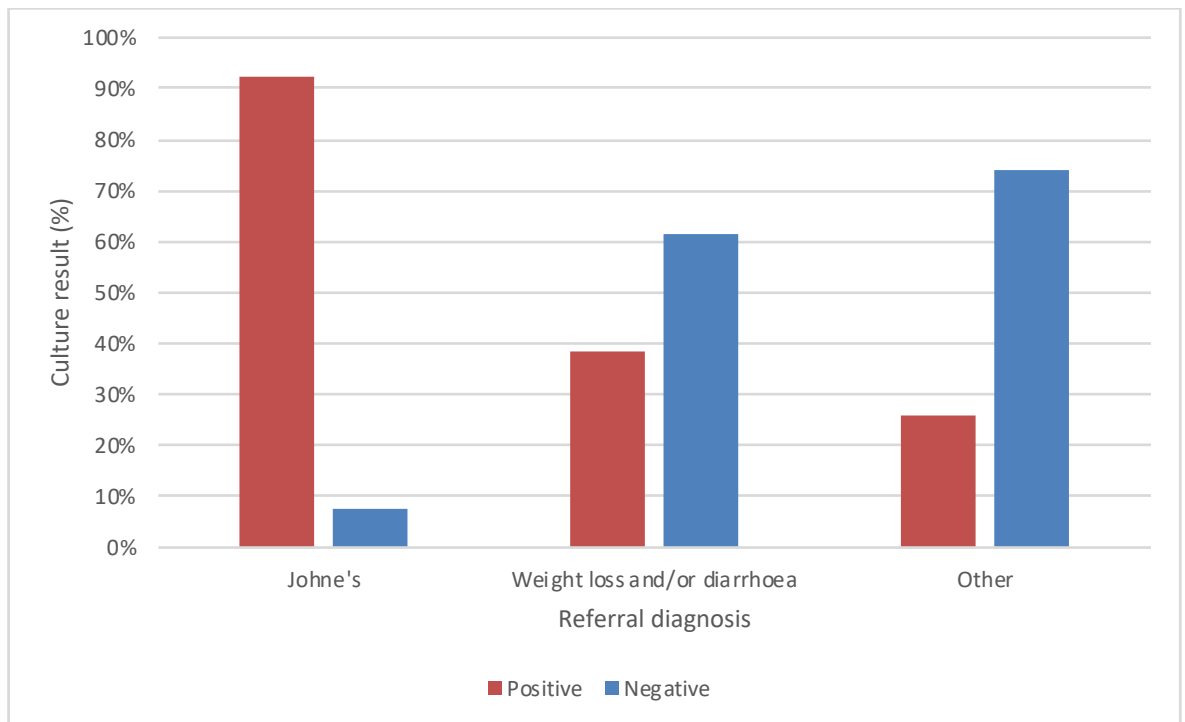
**Figure 3–9 Culture results shown by the referring vet diagnosis / clinical signs reported on the referral.**

## 3.3 Signalment

From the 49 animals that had a result for faecal culture, a total of 44 females and 5 males were sampled. Two males were positive and three tested negative on culture and half of the females (22 animals) tested positive on culture. Twenty-three dairy animals were tested and 11 were positive and 12 tested negative on culture. From the 26 beef animals tested, half (13 animals) were positive (Figure 3-10). Neither sex, nor breed purpose seemed to be correlated to a culture positive outcome (p=0.671) and (p=0.879) respectively.
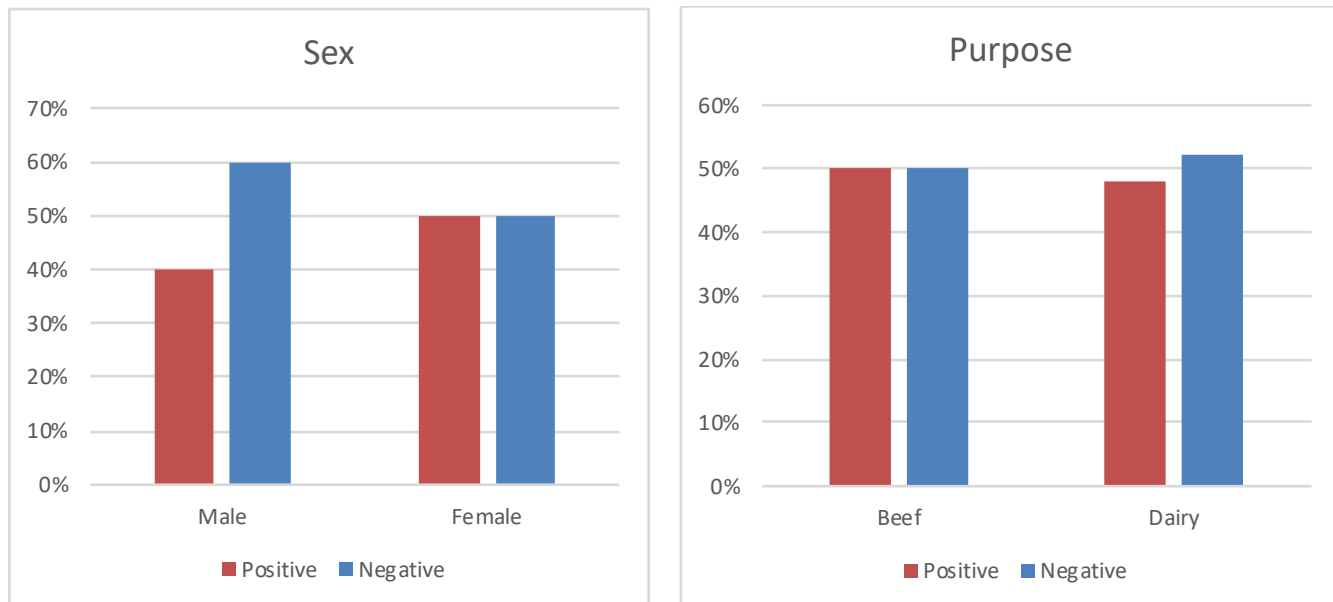
**Figure 3–10 Culture result shown by sex and breed purpose.**

Culture results grouped by breed showed that Holstein and Jersey were the most affected breeds in absolute numbers. For complete information of all the animals referred by breed see Appendix X. The percentage of Holstein cows positive on faecal culture was much lower compared to the Jerseys, where 6/6 were found to be positive on faecal culture. All three British Blue and the Belgian Blue animals were also all confirmed MAP positive by culture (Figure 3-11). It is important to note that five out of six Jersey cows came from the same farm. This farm had bought in animals from four different Jersey herds and had reported clinical Johne's disease cases on farm.
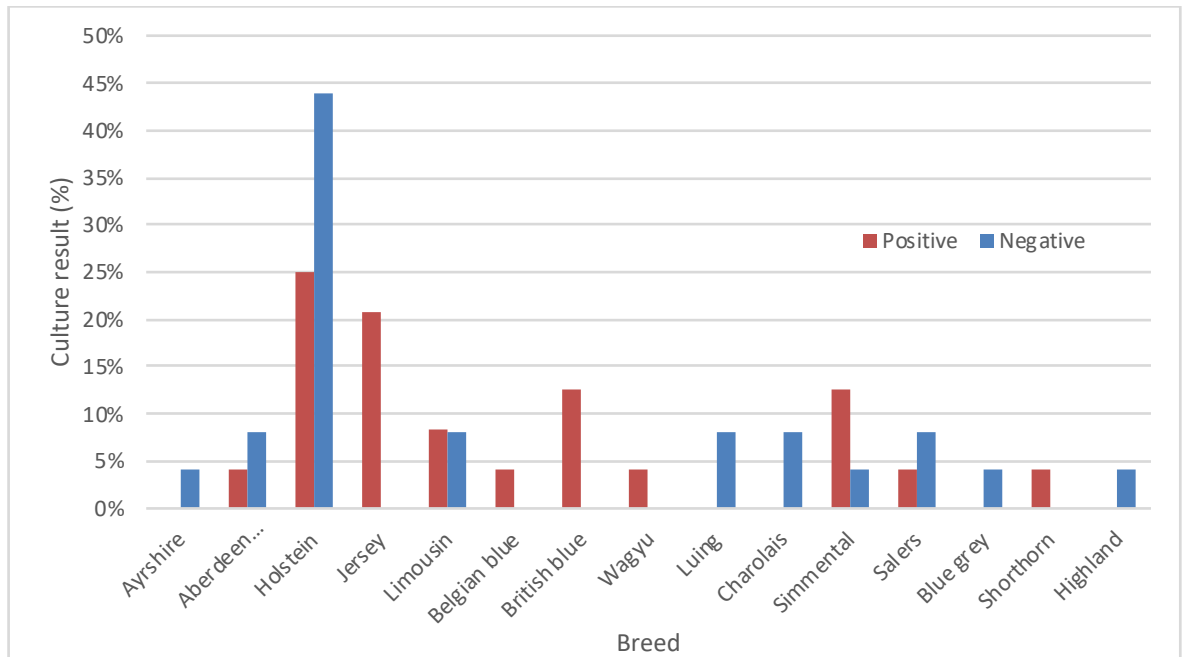
**Figure 3–11 Distribution of culture results (positive / negative) shown by the breed of the animal.**

The age of animals tested ranged from nine months of age up to 15 years old. The majority of the animals with a positive culture result were from two to seven years of age (83% of the total positives). Although two animals tested positive within the first year of age and two more animals were positives at older ages (Figure 3-12).
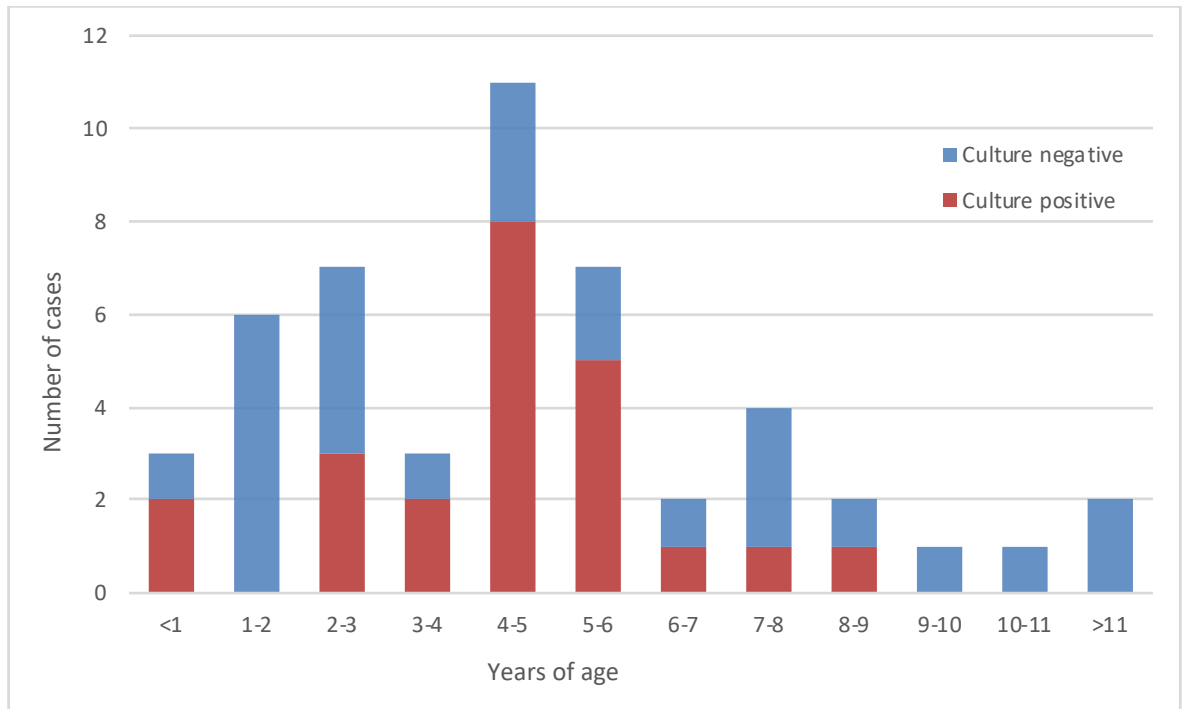
**Figure 3–12 Culture result shown by the age distribution**

## 3.4 Clinical examination data

A total of 49 animals met the inclusion criteria and had ELISA, PCR and culture results. For the purpose of data analysis, the clinical signs were grouped as:

- Animals met the inclusion criteria with no additional clinical signs

- Animals met the inclusion criteria but had additional clinical signs (e.g. respiratory disease signs, abnormal rectal examination findings, left sided abdominal ping, etc.)

From the group that showed no additional clinical signs, the majority of the animals (18/22) were culture positive (Table 3-1). The positive predictive value (PPV) of the clinical exam was calculated to be 82%. In contrast, from the animals that had additional clinical signs on clinical exam, 21/27 were culture negative. The negative predictive value (NPV) of the clinical exam was calculated to be 78%. A positive correlation was seen between animals showing clinical signs consistent with Johne's disease and a positive culture result (OR=15.75; 95% CI= 3.8332 – 64.7138). This meant that clinical exam findings were significant in regards to the Johne's culture outcome (p<0.001).

**Table 3-1 Clinical exam findings in relation to culture result.**

| | Culture result | | |
| --- | --- | --- | --- |
| Clinical Exam | Positive | Negative | Total |
| Consistent with Johne's disease with no additional findings | 18 | 4 | 22 |
| Consistent with Johne's disease with additional findings | 6 | 21 | 27 |
| Total | 24 | 25 | 49 |

Sensitivity: 75%, Specificity: 84%, PPV: 82%, NPV: 78%

It is worth mentioning some further test details of the four animals that were negative on faecal culture but had no additional clinical exam findings. These animals all tested negative on serology, 2/4 were PCR positive, 3/4 were younger than 2 years old and PM exam did not find any lesions compatible with Johne's disease in any of the cases. With regards to the six animals that had additional findings on clinical exam but were Johne's disease positive, 4/6 were PCR positive, the same proportion, but different individuals 4/6 were serology positive. Two animals had chronic pneumonia, two others had a left displaced abomasum (LDA) and another two had localised peritonitis.

### 3.4.1 Body condition score

The body condition score (BCS) on a 1-5 scale of 49 animals was compared to results from culture. From the cases that were positive on culture, a median BCS of 1/5 was obtained. In the negative group, the median BCS was of 1.5/5 (Figure 3-13). A difference of 0.5/5 BCS was present between the two group medians. This difference was not significant (p=0.477).
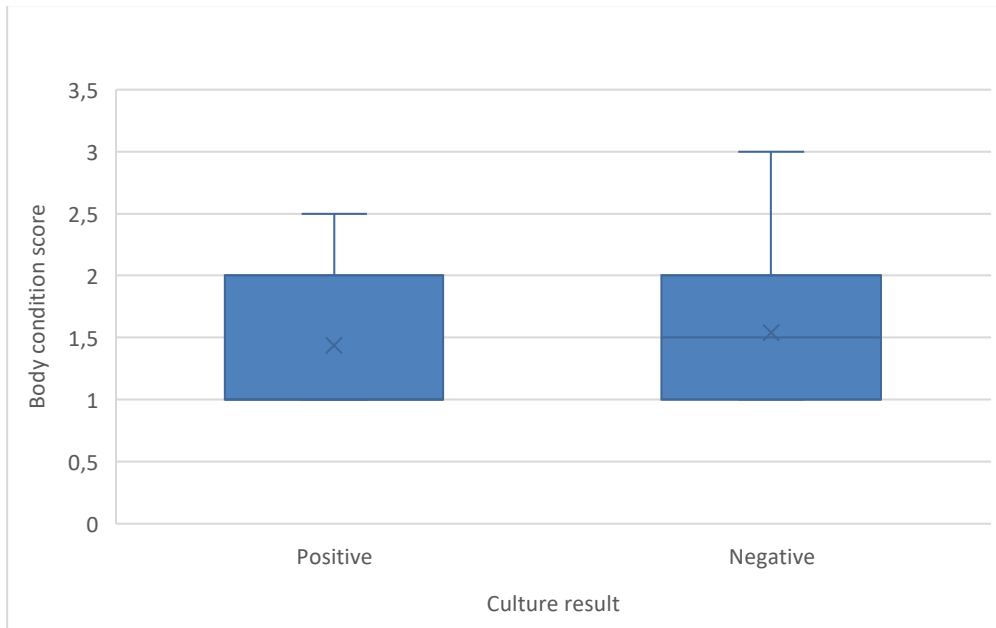
**Figure 3–13 Box plot of the Body condition score values (1-5) shown by culture results.**

## 3.5 Laboratory tests

### 3.5.1 Albumin concentration

From the 46 animals that were tested for blood albumin concentration and had a culture result, six had a low albumin. From those, three cattle had a positive culture result. The remaining 40 cattle had normal or high concentrations of albumin (Figure 3-14). Three animals had albumin concentrations over 34 g/L. There was no difference between animals that tested positive on culture and culture negative animals with respect to blood albumin concentration (p=0.891). Thus, albumin concentration did not appear related to the culture result.
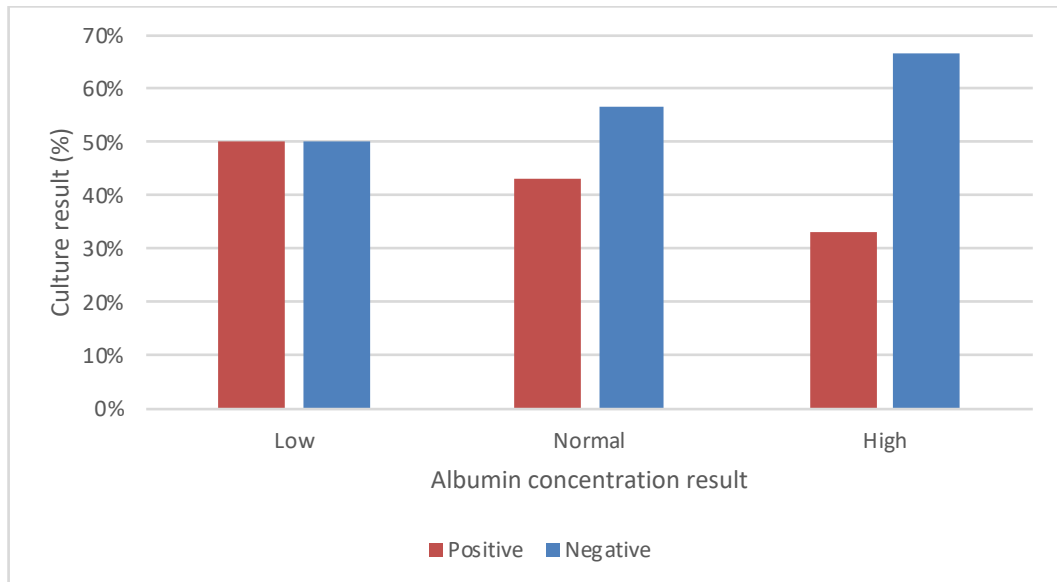
**Figure 3–14 Culture results shown by albumin concentration results. Low albumin concentration (<21 g/L), normal albumin concentration (21- 34 g/L), high albumin concentration (>34 g/L).**

When grouped by culture result, the albumin concentrations of animals that were Johne's positive on culture were not significantly different from animals that tested negative (p=0.643) (Figure 3-15).
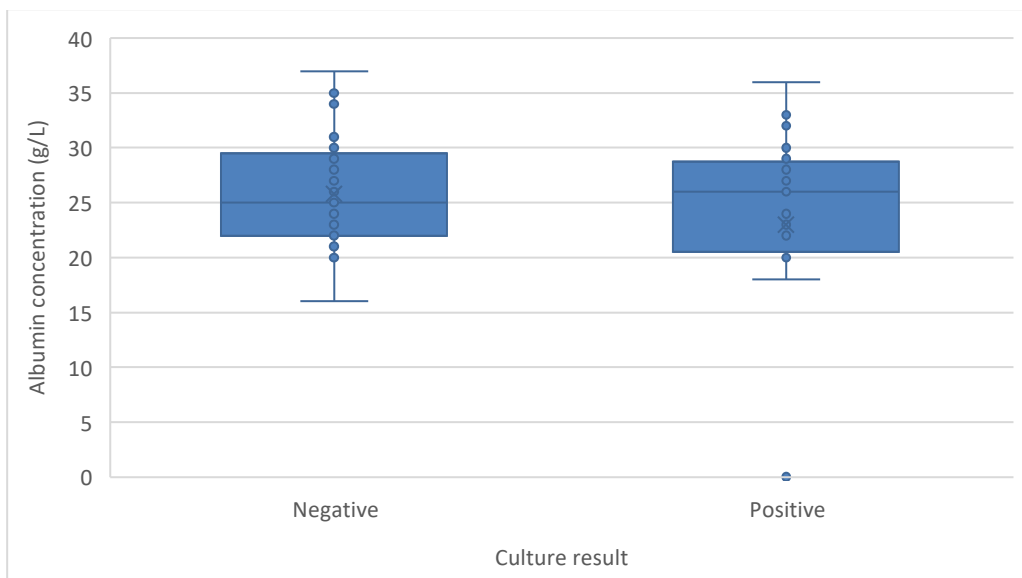


**Figure 3–15 Box plot of the albumin concentration (g/L) shown by culture result.**

## 3.5.2 Globulin concentration

A total of 46 animals were tested for blood total globulin concentration. From 28 cattle with normal globulin concentrations, 18 were positive on culture and 10 tested negative. Although two animals with high globulin concentrations tested positive on culture, the rest of the animals with high globulin concentration (16/19) were culture negative (Figure 3-16), the negative predictive value of blood globulin concentration was calculated to be 89%. A positive association was seen in animals with normal globulins and a positive culture result (OR=14.40; 95% CI= 2.7357 – 75.7975). Thus, normal globulin concentration was positively associated with Johne's culture outcome (p<0.001).
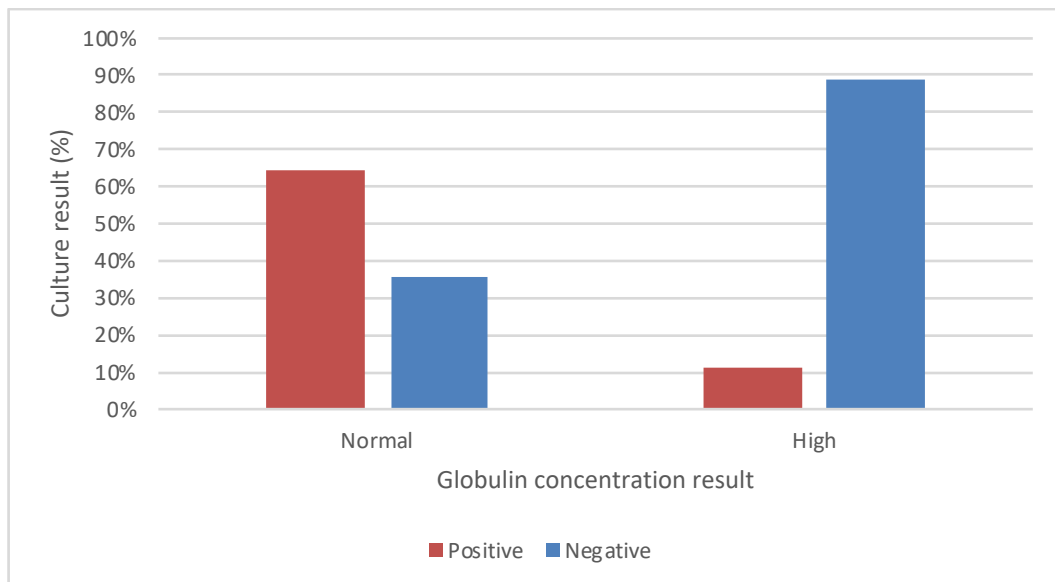


**Figure 3–16 Culture results shown by globulin concentration results. Normal globulin concentration (29-56 g/L), high globulin concentration (>56 g/L),**

When grouped by culture result, the globulin concentrations of animals that were Johne's positive on culture were significantly different from the animals that tested negative on culture (p=0.002) (Figure 3-17).
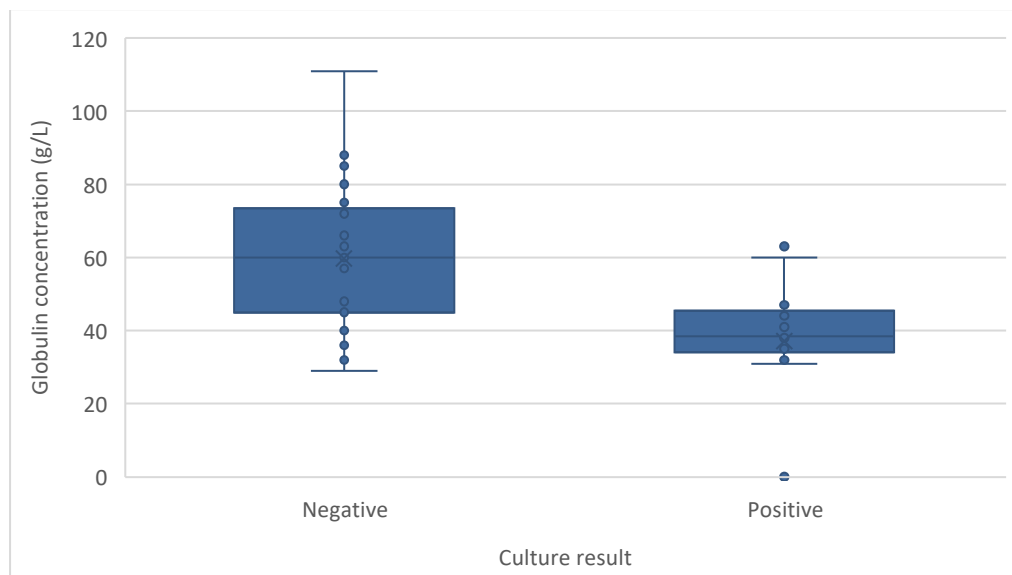
**Figure 3–17 Box plot of the blood globulin concentration (g/L) shown by culture result.**

## 3.6 Johne´s diagnostic tests

A total of 49 cases were tested for serology, PCR and culture, with 24/49 testing positive on faecal culture. However, results from the other diagnostic tests differed from the ones obtained from culture, the "gold standard test" (Table 3-2). Clinical examination findings consistent with Johne's disease were found in 23 cases, serology had 20 positive results, PCR and culture were positive in 24 cases

**Table 3-2 Summary of clinical exam and laboratory test results**

|  | Clinical exam | Serology | PCR | Culture |
|---|---|---|---|---|
| Consistent with Johne's / Positive test result | 23 | 20 | 24 | 24 |
| Consistent with Johne's but with additional findings / Negative test result | 26 | 29 | 25 | 25 |
| Total | 49 | 49 | 49 | 49 |

Culture identified 24 Johne's positive cases (Figure 3-18) from the total of 49 that were tested. Taking culture results as the gold standard, serology and PCR identified correctly 20 positive cases each respectively. There were four cases that tested positive on culture and were negative on serology (false negatives). From those, three cases were also PCR negative and one was positive. One animal had a negative result on PCR, but a serology and culture positive results.

All animals that were negative for culture (25/49) also tested negative on serology, which means that no false positive results were obtained from the serology test. Although four cases that were negative on culture had a positive result on PCR (Figure 3-18). These cases also had a negative serology result. Considering this, these four PCR positive cases would be classed as false positives. Taking culture result as the gold standard, PCR had four false negative results in relation to culture.
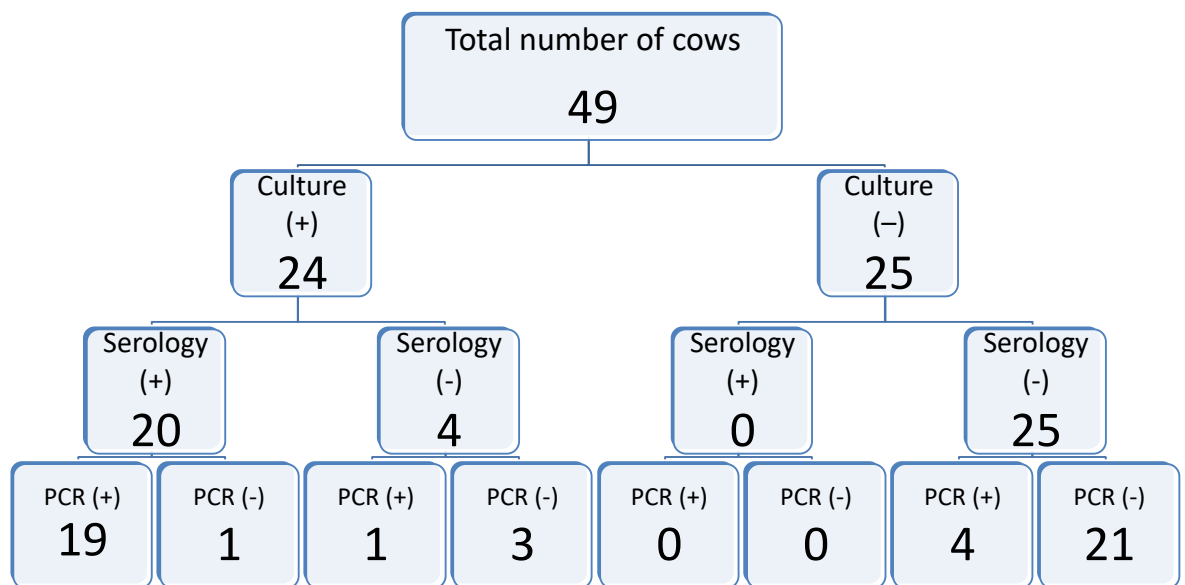


**Figure 3–18 Dichotomic graph showing the results of the serology and PCR tests in relation to faecal culture results.**

If we focus on the negative results of these three tests (Figure 3-19), of the 29 seronegative animals, culture was also negative in 25/29 (86%) of the cases. On the other hand, of the 25 PCR negative animals, culture was also negative in 21/25 (86%) of the cases. Twenty-one animals had a negative result in all the three tests.
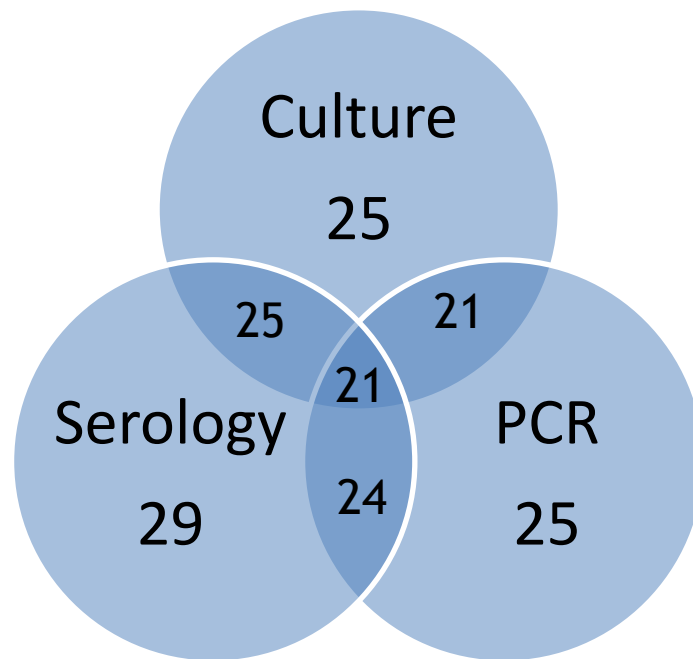
**Figure 3–19 Simplified Venn diagram showing the correlations within negative test results. Numbers displayed under the test name relate to the number of negative results for each test. The numbers in the intersections relate to the number of animals that had both negative results. The number in the middle shows the number of animals that had a negative result in all three tests.**

Focusing on the positive results of the tests (Figure 3-20). Of the 20 seropositive animals, culture was also positive in 20/20 of these animals 100%. On the other hand, of the 24 PCR positive results 20/24 were also culture positive (83%) and 19 animals had a positive result in all 3 tests.
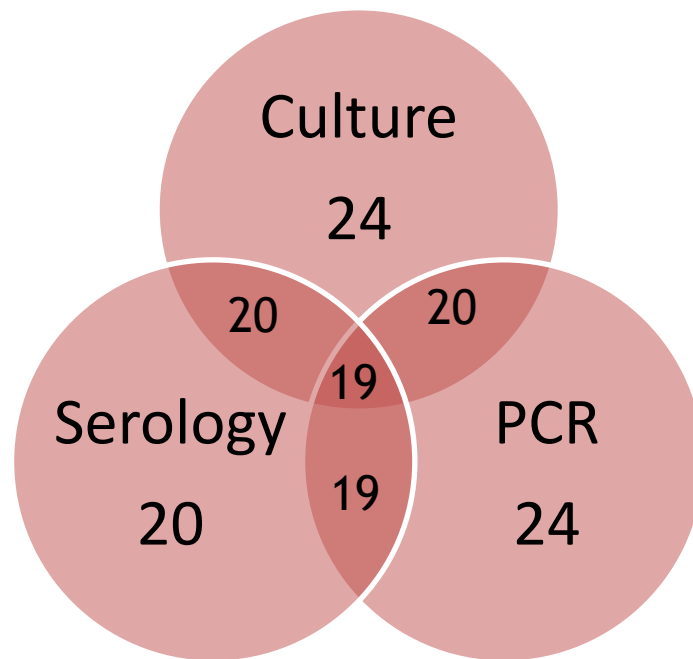
**Figure 3–20 Simplified Venn diagram showing the correlations within positive test results. Numbers displayed under the test name relate to the number of positive results for each test. The numbers in the intersections relate to the number of animals that had a positive result on both tests. The number in the middle shows the number of animals that had a positive result in all three tests.**

## 3.6.1 Serology results

Correlation between positive serology and positive culture tests (Table 3-3) was 83% (sensitivity), which meant that just four cases were not detected by serology in comparison to culture (false negatives, see details in Table 3-4). Negative PCR results correlated in 100% of culture negative cases (specificity). All serology positive results were confirmed as positive results by culture (20/20), meaning a positive predictive value of 100% in the present study (Table 3-3). Four cases that were negative in serology were found to be positive on culture, meaning that 25/29 negative cases were true negatives, making the negative predictive value of 86%.

A strong positive correlation was seen in animals that were serology positive and culture positive animals (OR=125; 95% CI= 12.9292 – 1208.5011). Significant differences on culture result were seen between seropositive and seronegative groups (p<0.001).

**Table 3-3 Culture results shown by the serology result.**

| | Culture | | |
|---|---|---|---|
| Serology | Positive | Negative | TOTAL |
| Positive | 20 | 0 | 20 |
| Negative | 4 | 25 | 29 |
| TOTAL | 24 | 25 | 49 |

Sensitivity: 83%, Specificity: 100%, PPV: 100%, NPV: 86%

The four cases that were serology negative but culture positive had different conditions at PM exam such as masseter atrophy, chronic pneumonia, chronic liver fluke and localised peritonitis, which could explain the clinical signs presented (Table 3-4). In addition, none of these animals had lesions suggestive of Johne's disease on PM examination and no acid-fast bacilli could be seen on the two cases that were sampled. Two of these animals were younger than one year old.

**Table 3-4 Detail of the cases that were Culture positive and serology negative. In this study, assumed serology false negatives.**

| Signalment | History (Hx) and Clinical exam (CE) in addition to inclusion criteria | Serology result and percentage of positivity (%) / PCR result | PM diagnosis / Histology |
|---|---|---|---|
| Jersey<br><br>Female<br><br>3 years 8months old | Hx: Known positive farm<br><br>CE: No additional clinical signs | Serology: Negative (0%)<br><br>PCR: Negative | Masseter atrophy, *Ostertagia* spp. and endometritis.<br><br>Histology negative |
| Aberdeen angus<br><br>Male<br><br>9 months old | Hx: poor growth<br><br>CE: Hind limb ataxia | Serology: Negative (0%)<br><br>PCR: Negative | Chronic pneumonia.<br><br>Histology negative |
| Limousin<br><br>Female<br><br>10 months old | Hx: Known positive farm, suspected VSD<br><br>CE: No additional clinical signs | Serology: Negative (37%)<br><br>PCR: Negative | Chronic liver fluke<br><br>Histology not sampled |
| Holstein<br><br>Female<br><br>4 years 5 months old | Hx: melena and abortion<br><br>CE: Metritis, atrial fibrillation | Serology: Negative (1%)<br><br>PCR: Positive<br><br>35.66 CT value | Cranial localised peritonitis<br><br>Histology not sampled |

### 3.6.2 PCR results

Correlation between positive PCR and positive culture tests (Table 3-5) was 83% (sensitivity), which meant that just four cases were not detected by PCR in comparison to culture (false negatives, see details in Table 3-7). Negative PCR results correlated in 81% of culture negative cases (specificity). Although, four cases were positive for PCR, but negative for culture (gold standard) which are classified as false positive results. From the 20 PCR positive results, 24 were confirmed as positive results by culture, meaning a positive predictive value of 83% in the present study (Table 3-3). Four cases that were negative on PCR were found to be positive on culture, meaning that 21/25 negative cases were true negatives, making the negative predictive value of 84%.

A strong positive correlation was seen in animals that were PCR positive and culture positive animals (OR=26.25; 95% CI= 5.7690 – 119.4422). Significant differences in culture result were seen between PCR positive and PCR negative groups ($p<0.001$).

**Table 3-5 Culture results shown by the PCR result.**

| | Culture | | |
|---|---|---|---|
| PCR | Positive | Negative | TOTAL |
| Positive | 20 | 4 | 24 |
| Negative | 4 | 21 | 25 |
| TOTAL | 24 | 25 | 49 |

Sensitivity: 83%, Specificity: 84%, PPV: 83%, NPV: 84%

The four cases that were PCR positive and culture negative were also negative for serology and had different conditions at PM exam such as pituitary cyst, chronic pneumonia, chronic liver fluke and eosinophilic enteritis (see details in Table 3-6). Three of these animals were younger than 2 years old and the other

one was 11 years old. In addition, the PCR CT values of these cases ranged from 33.9 to 38.0, which indicate a low MAP burden. The animal that was diagnosed with eosinophilic enteritis was recorded as with lesions compatible with Johne's disease on PM exam. After histopathology exam performed by one of the pathologist, as part of the normal diagnostic process on the farm animal hospital, these animals were diagnosed with eosinophilic enteritis. Lesions on the haematoxylin-eosin and Ziehl-Neelsen stain showed extensive lesions, but failed to find any acid-fast bacilli on the multiple intestinal sections examined under the microscope.

**Table 3-6 Details of the cases that were Culture negative and PCR positive.**

| Signalment | History (Hx) / Clinical exam (CE) in addition to inclusion criteria | Serology result and percentage of positivity (%) / PCR CT values | PM diagnosis / Histology |
|---|---|---|---|
| Ayrshire<br><br>Female<br><br>1-year 10 months old | Hx: Referred for blindness<br><br>CE: poor BCS and diarrhoea | Serology: Negative (0%)<br><br>PCR Ct: 35.84 | Pituitary cyst<br><br>Histology not sampled |
| Limousin<br><br>Male<br><br>1 year 2 months old | Hx: poor growth<br><br>CE: Chronic pneumonia | Serology: Negative (8%)<br><br>PCR Ct: 33.87 | Chronic pneumonia.<br><br>Histology negative |
| Luing<br><br>Female<br><br>11 years 9 months old | Hx: weight loss and teeth problem<br><br>CE: Dysphagia | Serology: Negative (0%)<br><br>PCR Ct: 38.02 | Chronic liver fluke<br><br>Histology negative |
| Simmental<br><br>Female<br><br>10 months old | Hx: chronic diarrhoea<br><br>CE: poor BCS and diarrhoea | Serology: Negative (0%)<br><br>PCR Ct: 37,67 | Eosinophilic enteritis<br><br>Histology negative |

**Table 3-7 Details of the cases that were Culture positive and PCR negative.**

| Signalment | History (Hx) / Clinical exam (CE) | Serology result and percentage of positivity (%) | PM diagnosis / Histology |
|---|---|---|---|
| Jersey<br><br>Female<br><br>5 years old | Hx: Referred with a Johne's suspicion<br><br>CE: LDA and chronic pneumonia | Serology: Positive (215%) | Bronchopneumonia<br><br>Histology negative |
| Jersey<br><br>Female<br><br>3 years 8 months old | Hx: Referred with a Johne's suspicion<br><br>CE: poor BCS and diarrhoea | Serology: Negative (0%) | Masseter atrophy<br><br>Histology negative |
| Aberdeen angus<br><br>Male<br><br>9 months old | Hx: poor growth, premature<br><br>CE: turaco-lumbar lesion | Serology: Negative (0%) | Bronchopneumonia<br><br>Histology negative |
| Limousin<br><br>Female<br><br>10 months old | Hx: chronic diarrhoea<br><br>CE: poor BCS and diarrhoea | Serology: Negative (37%) | Chronic liver fluke<br><br>Histology not sampled |

When grouped by culture result, the animals that were culture negative had higher PCR CT values than animals that were culture positive (Figure 21). CT values were significantly different between the two groups (p=0.007), which indicates that a higher burden of MAP was detected in samples that tested positive for culture.
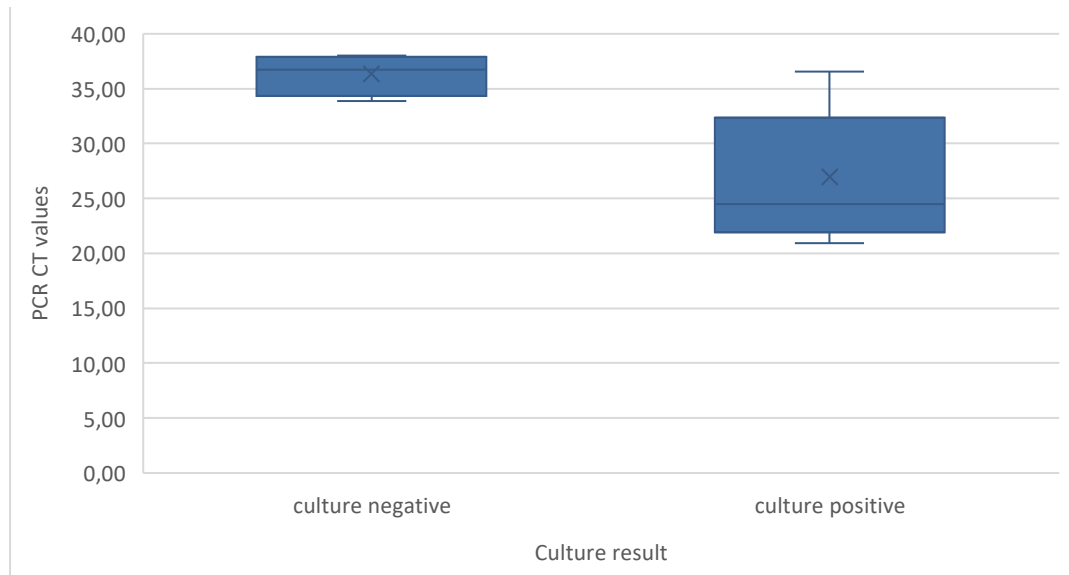


**Figure 3–21 Box plot graph of the CT values obtained by a direct PCR of faeces shown by culture result.**

# 3.7 Post mortem diagnosis and histology results

From the 49 animals tested for Serology, PCR and culture, only 33 had samples collected at post mortem examination for histopathology (Table 3-8). At post-mortem examination, 16/33 had gross lesions suggestive of Johne's disease (Figure 3-22). Histopathology examination detected lesions consistent with Johne's disease in 17/33 of the animals sampled. These animals had microscopic lesions showing the presence of acid-fast bacilli within the macrophages in the lamina propria or granulomas in the intestines of the animals examined (Figure 3-24).

**Figure 3–22 Picture of the intestines of the case 263444, showing severe thickening and corrugation of mucosa of the ileum (*).**

**Table 3-8 Summary of the results from cases that had full data set - clinical exam, PCR, culture, serology, gross pathology in post-mortem examination and histopathology examination.**

|  | CE | PCR | Culture | Serology | PM | Histo |
|---|---|---|---|---|---|---|
| Consistent with Johne's / Positive result | 18* | 20 | 20 | 18 | 16 | 17 |
| Consistent with Johne's with additional signs / Negative result | 15** | 13 | 13 | 15 | 17 | 16 |
| Total | 33 | 33 | 33 | 33 | 33 | 33 |

(*) Corresponding to animals classified into group I on the clinical exam. (**) Corresponding to animals classified into group II on the clinical exam. (CE) clinical exam; (PM) post mortem; (Histo) histopathology.

Results from post-mortem examination showed one false positive, corresponding to an eosinophilic enteritis case, which gives a specificity of 92%. Although the sensitivity of this test was lower (75%), due to five false negative results (Table 3-9). A positive correlation was seen in animals that had lesions suggestive of Johne's disease and culture positive animals (OR=36.00; 95% CI= 3.6923 – 350.9976). Significant differences in culture results were seen between Johne's disease compatible lesions group and absence of Johne's disease lesions group (p<0.001).

**Table 3-9 Culture results shown by the post mortem examination result.**

| Post-mortem examination | Culture | | |
|---|---|---|---|
| | Positive | Negative | TOTAL |
| Positive | 15 | 1 | 16 |
| Negative | 5 | 12 | 17 |
| TOTAL | 20 | 13 | 33 |

Sensitivity: 75%, Specificity: 92%, PPV: 94%, NPV: 71%

From the 33 cattle tested for histopathology, all of the animals that were positive on histopathology examination were also positive on culture, which means that specificity of histopathology was of 100% (Table 3-10). On the other hand, from the 20 culture positive cases, three were negative at histopathology exam, giving a sensitivity of 85%. A strong positive correlation was seen in animals that were PCR positive and culture positive animals (OR=73.67; 95% CI= 6.8483 – 792.4277). Significant differences on culture result were seen between histopathology positive and histopathology negative groups (p<0.001).

**Table 3-10 Culture results shown by the histopathology exam result.**

| Histopathology | Culture | | |
|---|---|---|---|
| | Positive | Negative | TOTAL |
| Positive | 17 | 0 | 17 |
| Negative | 3 | 13 | 16 |
| TOTAL | 20 | 13 | 33 |

Sensitivity: 85%, Specificity: 100%, PPV: 100%, NPV: 81%

A total of 33 cows had sections of ileo-caecal valve and lymph node examined on histopathology and 17 were found to have acid-fast bacteria present. Microscopic examination of these two areas yielded the exact same results, which means that both tissues were equally sensitive in detecting MAP infection. All positive histopathology results were confirmed by culture (no false positives). Moreover, PCR and serology results also coincided with the positive histopathology results. From the 16 cases that were negative on histopathology, three cases were negative on culture and positive on PCR and three more cases were positive on culture but negative on PCR (Figure 3-23). Details of these cases with inconsistent culture and PCR results were detailed previously (Tables 3-6 and 3-7).
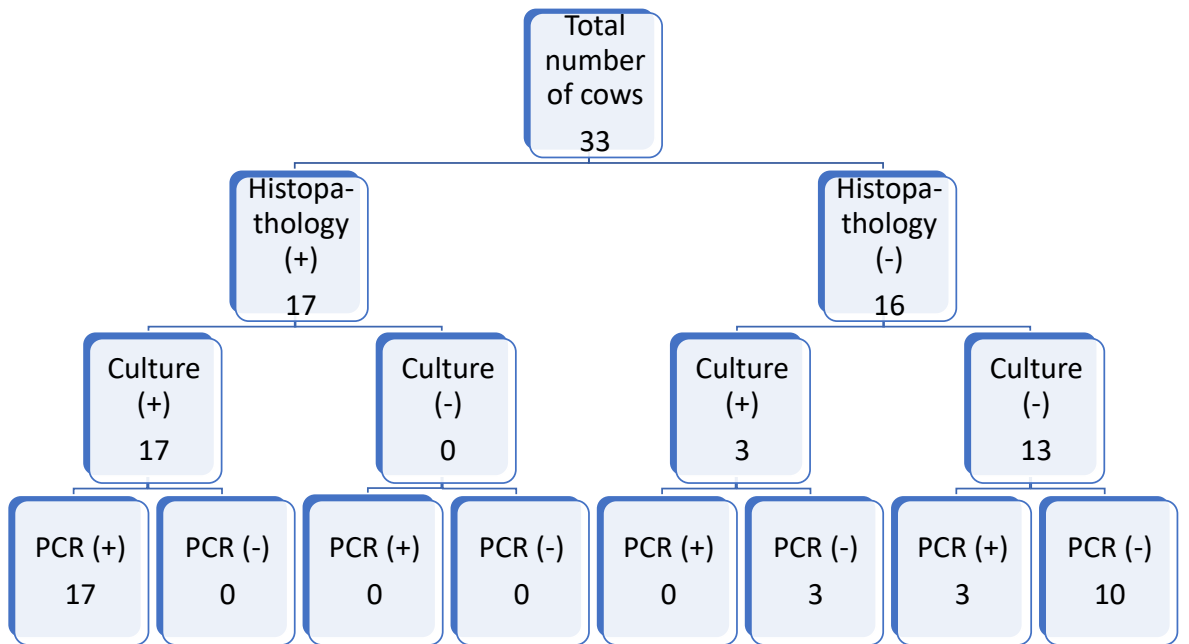
**Figure 3–23 Dichotomous diagram showing the results of culture and PCR tests in relation to histopathology results.**
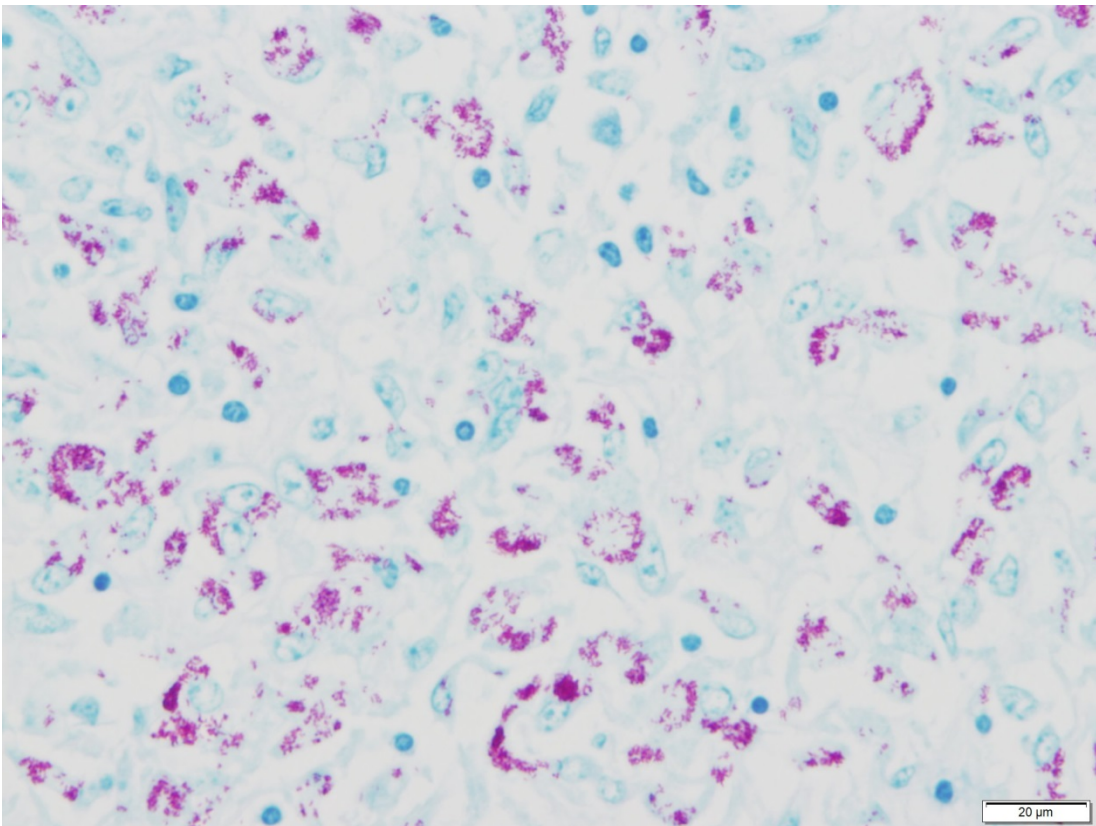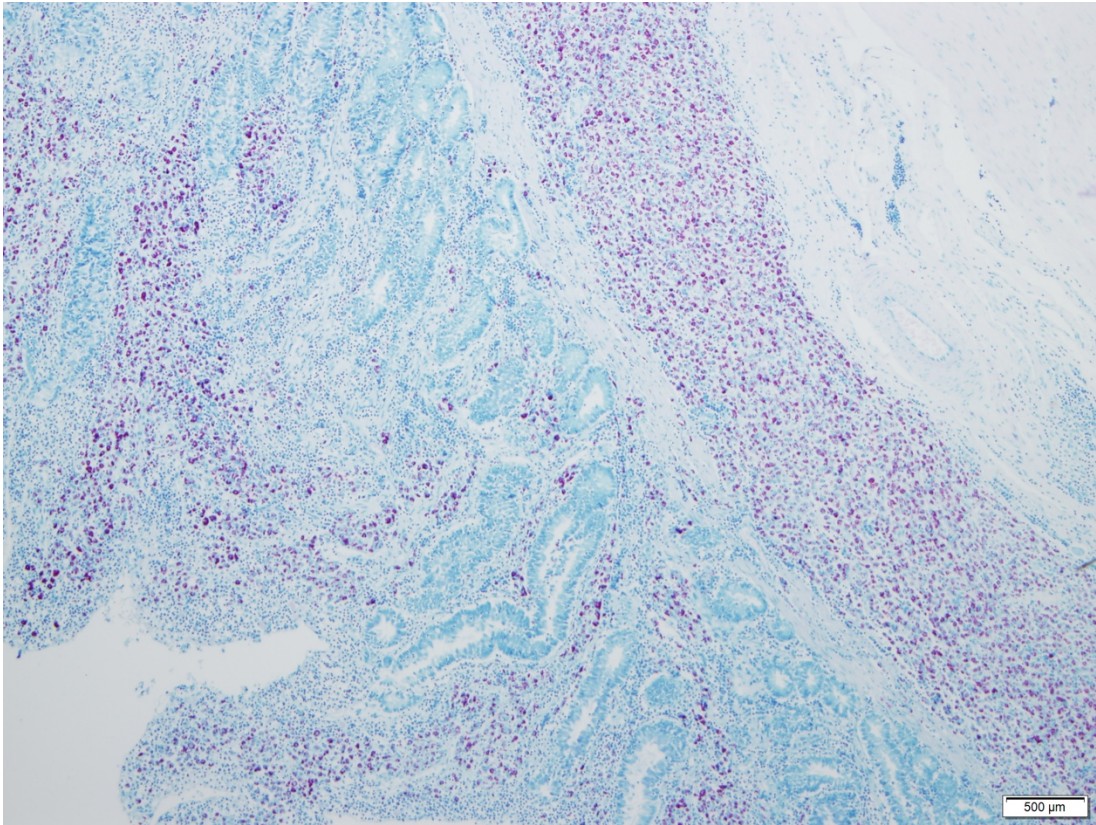
**Figure 3–24 Pictures of one section of the intestinal tissue proximal to the Ileo-caecal valve (overview on top, detail on the bottom) with acid-fast bacteria in the cytoplasm of macrophages, mainly in the lamina propria and mucosa.**

## 3.8 Liver fluke infestation status

A total of 46 animals had both a culture result and parasite lesion information recorded from post-mortem examination. Gross lesions compatible with fluke and presence of live adult fluke were recorded during post mortem examination (PME). A culture positive result was obtained in 23/46 animals and, from those, 11 had fluke lesions in the liver (Table 3-11). A complete absence of association between fluke lesions and culture results was found (p=1.00).

**Table 3-11 Culture results shown by fluke lesions found at PME.**

|  | Culture | | |
|---|---|---|---|
| Fluke lesions | Positive | Negative | TOTAL |
| Fluke lesions presence | 11 | 11 | 22 |
| No fluke lesions | 12 | 12 | 24 |
| TOTAL | 23 | 23 | 46 |

Three animals had concurrent live fluke at the time of PME (Table 3-12). Although, no significant differences were found between live fluke/no live fluke groups regarding culture outcome (p=0.265).

**Table 3-12 Culture results shown by live fluke found at PME.**

|  | Culture | | |
|---|---|---|---|
| Live fluke | Positive | Negative | TOTAL |
| Live fluke presence | 3 | 6 | 9 |
| No live fluke | 20 | 17 | 37 |
| TOTAL | 23 | 23 | 46 |

From the 26 negative serology results, 20 had no live fluke (Table 3-14) and 14 had no visible fluke-like lesions on PM examination (Table 3-13). One animal that was described with severe hyperplastic bile duct lesions also tested negative for serology (37%) and was positive on culture. This animal was 10 months old and also was also negative on PCR. Although no association could be found between groups (fluke lesions/no fluke lesions) and serology results (p=0.796).

**Table 3-13 Serology results shown by fluke lesions found at PME.**

|  | Serology | | |
|---|---|---|---|
| Fluke lesions | Positive | Negative | TOTAL |
| Fluke lesions presence | 10 | 12 | 22 |
| No fluke lesions | 10 | 14 | 24 |
| TOTAL | 20 | 26 | 46 |

Comparison between liver fluke lesion records and serology results were similar to the culture ones described above. No association could be found between live fluke/no live fluke PME group and serology results (p=0.494).

**Table 3-14 Serology results shown by live fluke found at PME.**

|  | Serology | | |
|---|---|---|---|
| Live fluke | Positive | Negative | TOTAL |
| Live fluke presence | 3 | 6 | 9 |
| No live fluke | 17 | 20 | 37 |
| TOTAL | 20 | 26 | 46 |

## 3.9 CT (cycle-threshold) values

Cycle threshold (CT) values from the PCR test conducted on liquid culture medium after 42 days of culture (culture CT values) were compared to CT values obtained from the faecal PCR. Results showed that faecal PCR CT values were less homogenous and with a higher median value (27.90) than the CT values obtained after 42 days on culture media, with a median of 19.70 (Figure 3-25). On average, there was a sixfold difference between faecal PCR and culture CT values (95%CI= 3.47701 – 10.4895) and this differences in CT values was statistically significant (p<0.001).
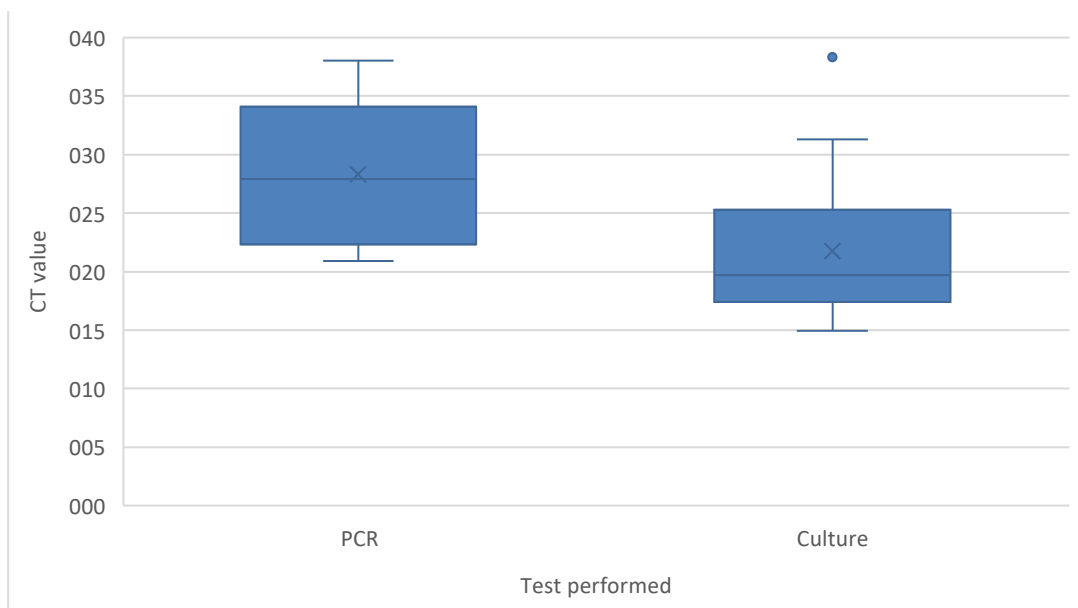


**Figure 3–25 Box plot of the CT values of PCR performed on faecal sample and the PCR done on the culture after 42 days.**

Culture CT values were in 15/16 cases, lower than those obtained from the PCR performed directly on the faeces, indicating that amount of MAP found in culture medium after 42 days of culture was higher than the amount of MAP detected on the same sample by direct faecal PCR. Within the culture group there was an outlier (with a CT value of 38.33) that was negative on PCR.

The animals that were PCR positive and serology negative had higher PCR CT values than the cases that tested positive for both PCR and serology. On average, there was a 10.18 CT value difference (95%CI= 4.08416 – 14.1892) between PCR positive/serology positive groups and PCR positive/serology

negative groups and this difference was statistically significant(p=0.002) (Figure 3-26). The cases that were PCR and serology positive had a bigger burden of MAP in faeces than the cases that where PCR positive and serology negative.
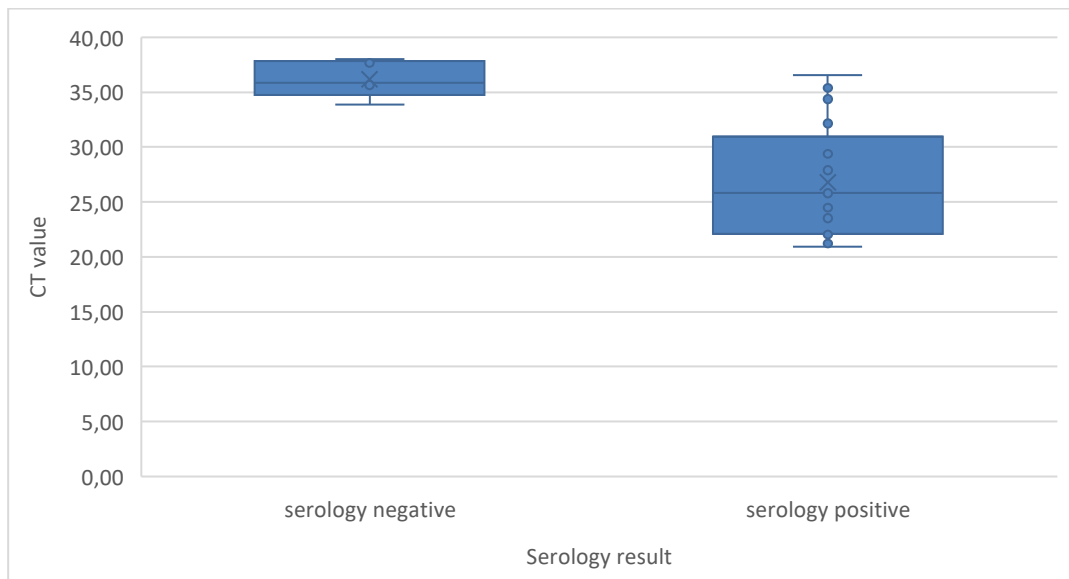


**Figure 3–26 Box plot of the CT values obtained by PCR done on faeces shown by serology result.**

Similar results to those seen with serology were also found with histopathology examination. The animals that were PCR positive and histopathology negative had higher PCR CT values than the cases that tested positive for both PCR and histopathology (Figure 3-27). On average, there was a 7.92 CT value difference (95%CI= 1.08402 – 13.4295) between the group that tested PCR positive – histopathology negative and the group that was positive on PCR and histopathology. Despite the differences shown, these differences were not statistically significant (p=0.096).
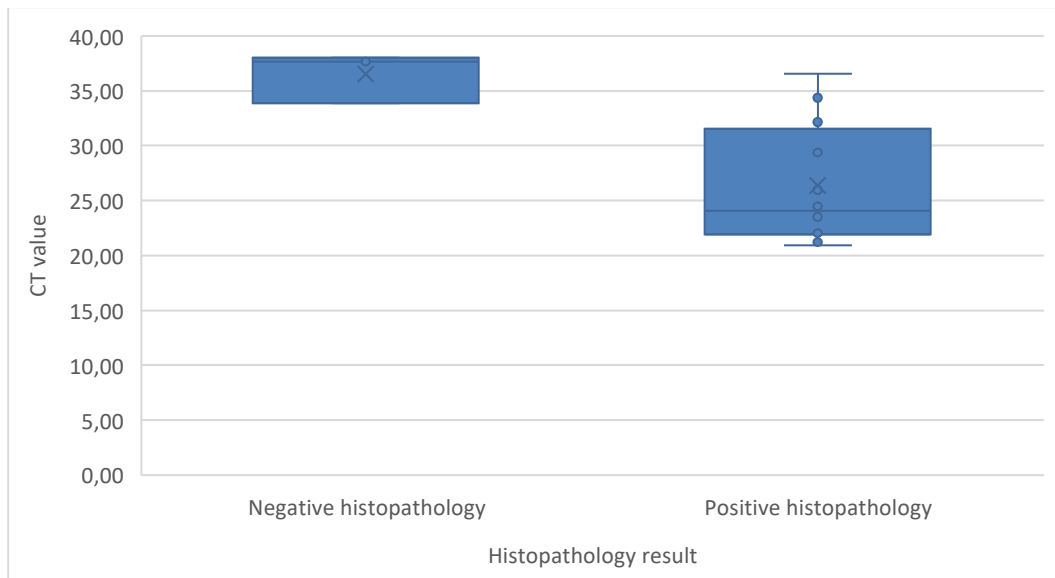
**Figure 3–27 Box plot of the CT values obtained by PCR performed on faeces shown by histopathology result.**

Correlation between serology percentage and PCR CT values (Figure 3-28) shows an association between the two values where the animals that have a higher serology percentage also show a significantly lower CT value on PCR (p<0.001). Despite this tendency, some animals show a high serology percentage of positivity, but also a high CT value. The fact that the correlation is not strong is also supported by the $R^2$=0.43.
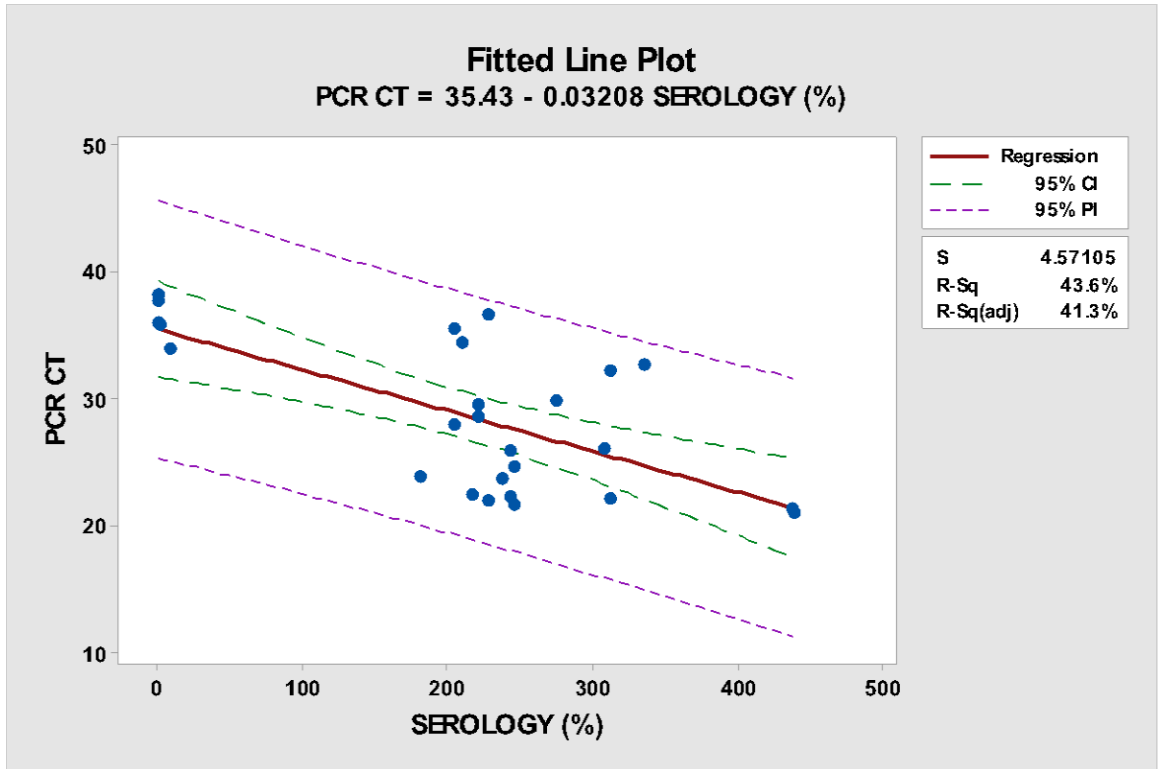
**Figure 3–28 Scatter plot and linear regression between the Serology (% values) and PCR-CT values.**

# Chapter 4    Discussion

Johne's disease is a chronic and wasting disease that affects cattle worldwide. Due to the long incubation period of the disease and its insidious nature, diagnosis of the disease is problematic. The main Johne's laboratory tests available in alive animals are serology, PCR and culture. Inconsistencies between test results are commonly seen in the field, particularly when sampling sub-clinically infected animals. This fact can lead to confusion and a possible failure to correctly identify the infectious status of individual animals. This can subsequently hamper MAP control at a farm and country level. The present work aimed to describe the history, clinical presentation and Johne's disease test results from animals that were submitted to a teaching hospital with clinical signs of Johne's disease. Furthermore, it aimed to analyse the test results and compare them to the current gold standard test – faecal culture.

This study allowed the in-depth analysis of farm or origin, history, clinical examination (CE) and diagnostic testing of cases suspected of having Johne's disease that were donated to a teaching hospital. It has highlighted some interested discussion points with regards to the diagnostic testing in particular, which could have an impact on the way these tests might be interpreted in future.

To the authors knowledge, this is novel work that included serology, PCR, culture and histopathology in each animal studied. Moreover, none of the previous studies, have included history, CE findings and gross pathology reports as part of the Johne's diagnostic process. The detailed information about the cases studied has been valuable in order to interpret the results obtained in this study.

## 4.1 Cattle signalment characteristics

### 4.1.1 Age

This study found that two animals at an unusually young age (nine and ten months old), tested positive on MAP faecal culture. This means that these calves shed viable MAP in faeces. These results agree with previous reported studies

(Mortier *et al.*, 2014[b]; Eisenberg *et al.*, 2015; Mortier *et al.*, 2015), where they suggest that MAP shedding by young calves could cause a calf to calf transmission and consequently be a meaningful infectious source of MAP in pen-grouped calves. Albeit these studies used high doses of MAP as they were experimental infection trials. The data from the current study suggest that MAP clinical signs and excretion can occur also in calves infected naturally in a commercial farm setting. The diagnosis of Johne's disease in youngstock that is reported in this study, should remind practitioners to include Johne's disease on the differential diagnosis list for calves with signs of diarrhoea, especially on farms where Johne's disease is present or suspected.

Calf testing has been suggested in the literature in order to detect MAP infected animals at an early age in order to exclude these MAP positive calves from breeding (Mortier *et al.*, 2015). In this study, calves inoculated at different ages test positive between one-two months after inoculation on culture and around four months after inoculation on serology. Histopathology positive results confirmed that these animals were infected. Although PCR or culture positive results in these animals would not predict if these animals would end up developing clinical signs. It seems that sampling animals at an earlier age could be a possible option in herd prevalence herds to try and identify infected animals sooner in order to make decisions regarding future replacement heifer potential earlier.

Calves showing clinical signs of Johne's disease at young ages have been linked to be exposed to a high MAP burden compared to animals that don't present with clinical signs until adults (Mortier *et al.*, 2013). Hence, detecting Johne's clinical signs in young animals should lead practitioners and farmers to suspect either that the calf's mother was a supershedder or, in cases where there is more than one young animal affected, that there is a high MAP burden on farm and implement urgent good husbandry practices to reduce Johne's incidence on farm. In addition, it would be necessary to carry out further investigations to find other causes of an early presentation of the disease. Other causes such as co-infections, synergism with other enteric pathogens or environmental factors would be interesting factors to study in order to understand why some animals develop the disease faster than others.

In the current study, two older animals (seven and eight years old) had also a positive culture results in an unusual advanced age. These animals were PCR and serology positive and had low albumin concentrations and oedema. This presentation fits the classical Johne's disease clinical picture that is described in the literature and text books (Van Metre *et al.*, 2008). However, this end-stage clinical presentation was not commonly seen on this study, which is probably either due to the culling of these animals on the basis of production losses before severe clinical signs are seen or due to "fit to transport" reasons. End-stage clinical signs can occur due to the chronicity of the disease or a "trigger" that could exacerbate the clinical signs in those animals. One common finding in both of these two animals was the presence of moderate-severe lesions of liver fluke, which could have exacerbated Johne's clinical signs or even triggered Johne's disease at an unusual advanced age (Naranjo Lucena *et al.*, 2017). In this article the authors suggest that liver fluke could suppress the cellular immunity (assumed protective) and shift it towards a humoral response (not protective). This hypothesis could explain the findings in these two old animals, but a co-infection trial should be carried out in order to explore this further.

## 4.2 Clinical examination and biochemistry

For the purpose of this study, clinical exam was analysed as a diagnostic test and compared to faecal culture as the gold standard. Sensitivity (Se) and specificity (Sp) were 75% and 84% respectively when compared to culture. This suggests that CE is an important part of the Johne's disease diagnosis process. Additional CE findings (i.e. peritonitis, ping on the left side or increased respiratory sounds) in an animal showing Johne's disease clinical signs (diarrhoea and poor body condition) reduced the chances of the animal being diagnosed with Johne's disease. Clinical examination sensitivity and specificity have not been calculated before to the author's knowledge. Thus, no comparison can be made with other studies. Probably, this is due to the difficulty to follow up cases from the time of suspicion of disease until the post-mortem examination (PME). In addition, the difficulty to report CE findings in a positive or-negative manner is challenging and could explain the absence of CE in previous studies.

Results from this study stress the importance of a comprehensive CE and accurate interpretation of findings by a veterinarian on all animals showing

clinical signs suggestive of Johne's disease. The CE should be performed in order to rule out other causes of poor body condition/weight loss and diarrhoea. There may be occasions where farmers recognise the classic signs of Johne's disease but rather than phoning their veterinarian to confirm, they decide to cull the animal on that basis alone. Reasons that could explain the lack of research in this subject might be the subjectivity of the CE process and/or the difficulty to interpret the additional clinical findings and decide if they could explain the diarrhoea and poor BCS/weight loss or not. Despite the relatively good sensitivity of the CE, precautions should be taken before extrapolating these results to the field or routine veterinary practice. The CE was performed in good handling facilities, it was complete and systematic and was done in most occasions with limited time constraints. Also, a minimum of three people (two students and a post-graduate student/clinician) examined the animal, which took on average 30 minutes to perform. In the authors opinion, all these factors increased the probability of detecting any additional and relevant clinical abnormalities. Most importantly, it is worth highlighting that results from the CE cannot be extrapolated to animals that are sub-clinically infected.

BCS performed at CE showed no correlation with the Johne's culture result. Johne's positive cattle had a median of 1/5 BCS where Johne's negative animals had a median of 1.5/5 BCS, differences were not significative. It is worth noting that due to case definition, all the animals in the study had poor BCS or a history of weight loss and diarrhoea. In addition, the lack of repeatability across investigators is also a limiting factor of this study. This may have hampered the evaluation of BCS as a predictive factor of Johne's disease in this study.

Three out of six animals had a BCS $\geq 2.5$ and were positive on MAP culture. These animals were included in the study due to a history of weight loss from the farmer. Three out of these six animals were also serology and PCR positive. These results highlight the importance of the history gathering from the practitioner and the need for a holistic approach when dealing with Johne's disease cases. Although, it could be argued that the history of weight loss from the farmer and vet could be vague (relying on memory or impression) with no actual body condition score numbers obtained from the history from the farmer or vet. It has been proposed that weight loss should be $\geq 10\%$ of the body weight

over one month (Whittington *et al.*, 2017) and this could not be proven in this study.

First line diagnostic tests such as basic albumin and globulin concentrations (which could be performed in an in-house laboratory) were studied and compared to the culture results. These basic parameters helped to better describe the cases; despite they are not novel in Johne's disease research, it is the first time that they have been combined with the other diagnostic tests and compared directly. The hypothesis was that the only indicator of Johne's disease would be albumin concentration. The results showed some unexpected outcomes, which will be explained below.

Johne's disease is described as a protein losing enteropathy and previous papers described occurrence of low total proteins (TP) and low albumin concentration (Clarke, 1997), but no proportions were stated. Hence, low albumin concentrations were expected in most of Johne's culture positive cases. However, in this study only six animals had a low albumin concentration and just three of them were Johne's culture positive animals. Dehydration status in some cases or early stage of the disease in other cases could potentially explain these results. Although, none of the animals had an increased PCV (data not shown) which would have been expected in a dehydrated animal. Sixteen cattle were mildly dehydrated (5 - 7.5%) and none of the animals presented with severe dehydration. In relation to this, there is no reported method to perform an albumin concentration correction in light of hydration status, so assessing albumin concentrations properly could have been problematic. Although, results showed that hypoalbuminemia does not occur in most of the Johne's cases as is classically assumed and thus, albumin would not be advised as an indicator of Johne's disease based on this study. A lack of research has been noticed in this area, hence further specific research would be necessary to clarify if albumin concentration values can be used by the practitioner in the field as a reliable indicator.

In addition, BCS and albumin correlated well with each other (data not shown). This correlation would probably just be indicative of the chronicity or the catabolic status of the animals (Clarke, 1997).

Data analysed from this study showed that Johne's disease culture positive animals tended to have normal concentrations of globulins. Only two animals showing high globulin concentrations were positive on culture, which meant that globulin concentration had a negative predictive value (NPV) of 89%. These results suggest that high globulin concentrations would indicate an inflammatory process that would explained the clinical signs shown by the animal. Globulins could be useful for the practitioner when examining an animal with ill-thrift and diarrhoea if basic biochemistry results were available. If normal concentrations are found it would be of little help, high concentrations would indicate that the animal probably has not got Johne's disease and is suffering from another inflammatory or infectious disease. To put this result in context however, if a practitioner suspects Johne's disease, serology would be a more reliable test, which is cheap and much more specific and sensitive than globulins in animals showing clinical signs (see below).

## 4.3 Serology results

Serology is described in the literature as a 'good' test in animals clinically affected by Johne's disease (Whitlock *et al.*, 2000; Weber *et al.*, 2009). In this study, sensitivity (87%) and specificity (100%) values were similar with a previous study by Weber *et al.* (2009), where the sensitivity and specificity of the serology in animals showing clinical signs was 83% and 99% respectively. Positive and negative predictive value were respectively 100% and 86%, which differ slightly to previously published work, where PPV and NPV were respectively 99.7% and 56.7% (Weber *et al.*, 2009), but these values vary depending on the prevalence of culture positive results and cannot be compared.

The four animals that had a false negative result on serology (taking culture as the gold standard) can be explained given the reported lower sensitivity of serology in comparison to culture (Whitlock et al., 2000). However, a negative result for serology in a culture positive animal could suggest that these animals were infected but not in the clinical stages of Johne's disease. Thus, the clinical signs shown could be due to other conditions. In order to understand the four false negative results, clinical case details for each animal were investigated. In all four cases there were no Johne's disease lesions at gross PME and no evidence of mycobacteria following ZN staining and histopathological exam of

tissues. In addition, these cases had additional findings on CE and PME which could explain the clinical signs of ill thrift and diarrhoea. These facts may lead us to hypothesize that these animals probably were in an early stage of the disease. Thus, Johne's disease could be a concomitant infection in these animals, but actually not being the major contributor to the signs seen. The fact that three of these animals had a negative result on PCR suggests that the MAP burden was low and a pre-clinical stage could be hypothesized in those cases. Another option could be a false positive of the culture result, the "gold-standard". Contamination of the sample during sampling or a gastrointestinal pass-through are the two main scenarios where a false positive could occur and they will be discussed below (see section 4.3.2 - Culture results).

The absence of false positive serology results in this study was also expected and agrees with the literature. The main factor that can result in a false positive results with serology is the intradermal tuberculin test. It is expected that this should be less common in the population sampled, as Scotland is officially BTB free and the frequency of BTB testing is very low, most herds being tested every 4 years (APHA, 2018), compared to other regions of the United Kingdom.

The cost of the serology test is approximately six times cheaper than the cost of PCR which in this study had yielded lower specificity values compared to serology (Table 1-1-Chapter 1). Culture is nine times more expensive than serology and has a much longer turnaround time (which can also contribute to more economic losses on the farm). Thus, it is worth highlighting the cost-benefit outcome of serology as a specific and valuable test. In conclusion, high sensitivity and specificity in animals showing clinical signs make serology a good cost-effective test. Its short turnover time (less than a week) makes it the most appropriate test in animals with suspected to be suffering from clinical Johne's disease on farm.

## 4.4 PCR and culture

### 4.4.1 Four false positive results on PCR

Taking Culture as the gold standard, the four cases that were culture negative and PCR positive are classified in this study as PCR false positive results. All four

cattle were also serology negative and had lesions at post-mortem examination (PME) that could explain the clinical signs shown (i.e. non-Johne's type lesions). Considering that the majority of the literature affirms that PCR has a specificity of 99% (Leite *et al.*, 2013; Prendergast *et al.*, 2018) and culture is the most sensitive test (Whitlock *et al.*, 2000), these results were not expected. Although, Laurin *et al.*, (2015) found also PCR positive-culture negative cases and suggested that in that study, PCR could have been more sensitive than culture.

PCR CT values of all four cases were high (33.87 – 38.02), which means that MAP burden in those faecal samples was low. A positive PCR but culture negative result can be explained by different reasons: it can indicate that MAP genetic material was present in the faeces, but MAP failed to grow after culture; that MAP was not distributed homogeneously throughout the faeces sample; that sample size used in culture and PCR was different; that laboratory steps for liquid culture systems could have harmed the survival of small number of MAP organisms.

Another hypothesis that could explain these results is a gastrointestinal pass-through process in these animals. This process has been described before by other authors (Hines *et al.*, 2007; Fecteau, 2018). In a high prevalence farm, with high-shedding cows in contact with non-MAP infected animals, PCR false positive were achieved (Sweeney *et al.*, 2012).This author suggests that even a culture result could be positive due to a pass-through process. The pass-through process has also been well described in studies where young calves were infected, in which the detection of MAP by PCR on faeces was attributed to a pass-through until seven days after exposure (Corbett *et al.*, 2017). The pass-through process adds complexity to interpretation of positive faecal results.

## 4.4.2 Culture results

False positive in culture is controversial, but theoretically possible. This was suggested with reasonable supportive data by Fecteau *et al.* (2010) and Sweeney *et al.* (2012), who proposed that culture could also yield a positive result due to a pass-through. Given the high MAP burden in the environment where the cattle from the current study were tested, this hypothesis is worth considering when analysing the present results. If it is true that culture testing is the most

sensitive and specific test in normal conditions, questions around false positives have been raised by some papers (Fecteau *et al.*, 2010; Sweeney *et al.*, 2012). Hence, it would be necessary to clarify the hypothesis of a positive culture result due to a pass-through process and establish a "gold standard" test in highly MAP contaminated environments.

The high MAP burden expected in the Farm Animal Hospital could have played a role in results obtained by increasing the chances that non MAP-infected animals would become in contact with MAP. As a result, incongruent results between PCR and culture could have been more probable in this study than in other settings as non-infected animals could pick up MAP from the Farm Animal Hospital environment and have a positive PCR result without actually being infected themselves. In an attempt to apply this to the field situation within a high MAP burden farm, an individual positive PCR result should be taken with caution. A positive PCR result would indicate that there is infection on the farm but there is still a possibility that the animal sampled is not the one actively shedding MAP.

Another option that could explain a PCR positive - culture negative result would be that culture has a lower sensitivity than published figures suggest, or a failure to culture MAP from specific cases. Although culture is described as the most sensitive test (Whitlock *et al.*, 2000), a strain factor could be causing an impaired growth on culture. De Juan *et al.* (2006) reported sheep strains (Type I/III) take longer to grow in culture. Such strains can also infect cattle, although from the four cases studied, all the animals came from farms with no sheep. Further research could be carried out in order to genotype the MAP strains that caused an apparent false positive on PCR. Alternatively, a series of PCR tests could be performed on the culture media in order to better understand how and when the negative result appears and then infer why it happens.

Further research could confirm false-positive culture results by exposing Johne's disease low risk adult cattle to a contaminated environment and compare faecal culture to other tests as tissue culture. If the faecal culture is positive and tissue culture is negative this study could suggest that not always a faecal culture positive result means that the animal is infected, but just that the animal is infectious, as suggested previously by Whittington et al. (2017). However, tissue

collections would be ethically difficult to support in live animals and highly costly if the tissues were collected post-mortem (animals should be culled for the purpose of the study). New diagnostic methods, such as the blood phage-based detection method or other tests could be used as additional tests to compare the culture results.

## 4.5 PCR CT value application in the field

Published studies in the literature have attempted to identify MAP super-shedders by using PCR CT values. Although correlations between PCR CT values and CFU (colony-forming unit) have been proven imperfect, there is a tendency for a higher number of CFU to be correlated with a lower PCR CT value in faeces (Aly *et al.*, 2012; Laurin *et al.*, 2015; Corbett *et al.*, 2018). Different cut-off CT values have been proposed in order to identify super-shedders. Aly *et al.* (2012) proposed a cut off of <25.9 CT value with a sensitivity of 73.7% and a specificity of 94.3%. In this study they defined super-shedders as >10,000 CFU/g of faeces. Another study performed by Laurin *et al.* (2015) proposed a cut-off value of <29 CT value with a sensitivity of 100% defining a super-shedder as >50 CFU/tube (test tube filled with HEYM culture media). Although both studies used the same PCR Kit, the super shedder CT cut-off value was different, which highlights the challenge of the CT value interpretation of the PCR Kits available nowadays. Moreover, Ct values from the present study are difficult to compare to those reported in the literature due to the use of different PCR kits and super-shedder definition., which makes it difficult to classify cases as high, medium or low shedders.

In order to differentiate active shedding from a pass-through shedding the CT values of the PCR have been proposed as a valuable tool (Corbett *et al.*, 2018). CT values could help to classify the animals in two groups: high MAP shedding (low CT value and likely to be an active shedding animal) or a low MAP shedding (high CT value and possible passive shedding animal). Hence, the CT values could help farmers and vets to make culling decisions when there are multiple positive animals on farm and culling them all at once is not an option; they can target culling of those animals in the high MAP shedding (low CT value) group. However, more work should be done by the PCR kit manufacturers in order to

achieve a reliable tool that would quantify effectively the level of shedding for each of the animals sampled.

Faecal shedding however is highly variable (Laurin *et al.*, 2015), which could misclassify some of the high-MAP shedders and lead to a failure of control of Johne's disease on a farm. To be able to use CT values more accurately it would be necessary to better understand MAP shedding patterns and MAP cycle of shedding (Koets *et al.*, 2015; Barkema *et al.*, 2017). Further work to clarify the proposed shedding cycle of 42 days should be carried out (Koets *et al.*, 2015). This information would allow us to sample an animal at different times and therefore obtain a more representative sample that could potentially be more informative of the MAP shedding level and even more sensitive than just a single faecal sample. A strategic faecal sampling at different time-points would increase the sensitivity and specificity of PCR test in a similar fashion as it is currently performed in the quarterly milk serology test in cows. However, repeated faecal sampling in some farms would be challenging as facilities, handling and time investment would make this option impractical. Furthermore, repeated sampling would have cost implications.

In the present study PCR CT values were also compared to serology results. The aim was to use serology percentage of positivity as a predictor of the shedding status of the animal. In order to achieve that, a correlation of PCR CT values and serology results were performed. The results obtained showed a tendency, for high serology percentage results to be correlated with low PCR CT values. The animals that had the higher serology results (>437%) had the lowest PCR CT values and the animals that had the highest CT values (>37) had a negative serology test. These results showed that in some occasions, serology results could help predict the shedding status of the animal. Although, positive serology results ranging from 180 - 335 had a poor relationship with PCR CT values ($R^2$=0.43) and thus cannot be correlated to a shedding status of the animal.

## 4.6 Gross post-mortem examination

Results obtained from the gross PME were more reliable when compared to culture than expected. To the author's knowledge, sensitivity and specificity data have not been reported in the literature. A sensitivity of 75% and specificity

of 92% from the PME was found. One factor that could have played a role in these results could be the advanced clinical stage of the animals included in the study, which would have resulted in more prominent lesions. Additionally, Johne's laboratory tests results (i.e. serology or PCR) were reported in the PM form to the pathologist before the PM was performed. Thus, PME was not a "blind test" and there is the potential that this information could have influenced the pathologists in their interpretation of PME Johne's lesions.

Another factor to take into account is that the majority of PM examinations were carried out on fresh carcasses euthanized approximately four hours before the necropsy. Furthermore, one of the cases that was euthanized the day before the PME had no clear lesions on the PM, but had positive results for serology, PCR, culture and histopathology. Hence it is reasonable to think that even mild autolysis of the enteric tract could impair Johne's disease lesion recognition. It is worth to mention that PME were performed by a group of final year students supervised by the pathologist in charge. Each week the students and pathologist were different following a rotational basis, so there was a high variability of assessors, which could have played a role in the results obtained. In conclusion, fresh state of the carcasses, previous result information and pathologist variability make that the repeatability of the results difficult. When trying to extrapolate these findings to the field, caution should be practiced, mainly due to factors such as facilities, autolysis of the animals, time availability and training required.

## 4.6.1 Eosinophilic enteritis

Within the 59 cases that met the inclusion criteria there were two animals that were diagnosed with eosinophilic enteritis. They were the only cause of a false positive result at PME. These animals were recorded to have lesions compatible with Johne's disease at PM, but further histology showed no MAP bacteria. One case was negative for culture and serology, but positive on PCR. The other case was positive on serology and PCR, but was not sampled for culture. There is no clear pathogenesis of eosinophilic enteritis reported in the literature and the general assumption is that is still idiopathic (Fushim*i et al. 2014;* Van Metre *et al.,* 2008). Some theories link it to parasite infestation, although there is no proof of it. Some other authors have observed some links between eosinophilic

enteritis and Johne's disease, suggesting that it can be an abnormal, exacerbated, presentation of the disease (Monif and Williams, 2015) and it could explain why these cases were both positive for Johne's using some of the other diagnostic tests. In the study by Monif and Williams, a series of 12 cases diagnosed with Johne's disease (based on PME lesions) were submitted for PM examination and histology examination. Some of these cases showed absence of acid-fast bacteria on ZN histopathology examination, the hypothesis stated in that paper was that an eosinophilic reaction could be effective at clearing MAP infection. It would have been ideal to perform culture or PCR test from the mucosa and lymph nodes of the slides with no MAP seen, this information would have been valuable tests to reach a definitive diagnosis. The small number of animals included in this study, the absence of tissue culture/PCR and the absence of further literature to support his hypothesis means further research will be needed.

## 4.6.2 Parasite status of the animals

Previous authors have hypothesised that liver fluke infection could be a predisposing/triggering factor for Johne's disease (Naranjo Lucena *et al.*, 2017). The results obtained in this study could not confirm such a hypothesis. Neither live fluke or liver fluke lesions correlated with a positive MAP result with either serology or culture. Naranjo *et al.*, (2017), suggested that liver fluke would reduce the cellular immunity of the host and accelerate the clinical signs of the disease. As the author already acknowledges in that paper, an experimental coinfection model would need to be developed and tested in order to prove such hypothesis.

## 4.7 Histopathology

Results obtained from histopathology yielded a specificity of 100% in relation to culture. These results were expected, but they are still remarkable. Even more, the two sites sampled, the ileo-caecal valve and ileo-caecal lymph node coincided completely and had the same result following histopathological examination.

The sensitivity of histopathology was unexpectedly high (85%) compared to the sensitivity reported previously (Martinson *et al.*, 2008). Previous research varied in sample size, animal age, infection models and site sampling, but sensitivity reported ranges from 0% to 100%(Huda and Jensen, 2003; Sweeney *et al.*, 2006). In the study where they achieved the highest sensitivity, the authors examined 18 sample sites taken from every one of the 16 cows examined (Huda and Jensen, 2003). Animals included in the study had different reasons to be culled, after repeated culture test on faeces five were positive and eleven were negative. Each site had two ZN slides examined and each slide was examined twice. Thus, histological work carried out in this study is difficult to replicate in other studies and even more difficult in a diagnostic commercial laboratory due to the time commitment involved. On the other hand, studies that have established histopathology sensitivity to be much lower. The study that achieved a 0% of sensitivity on histopathology (Sweeney *et al.*, 2006) should not be considered as a like for like comparison to this study as calves were sampled only three weeks after inoculation and at 42 days of life. In another study, histopathology had a lower sensitivity at slaughterhouse (5.3%) when compared to tissue culture (Martinson *et al.*, 2008).

The sensitivity from the present study is relatively high considering that only one sample was collected from each site and each slide was examined for 10 minutes. It is worth noting however, that said twenty cases that met the inclusion criteria did not have samples collected for histopathology. Fifteen histopathology samples were missed from animals that did not have lesions consistent with Johne's disease and seven samples were missed from animals with gross lesions found on the PME. Thus, the sample of the histopathology was not representative of all cases and results of sensitivity and specificity should be taken with caution. In addition, the ZN examination was performed by a non-pathologist, but trained researcher. Thus, repeatability of these results would be important to validate a simple, efficient, specific and prompt test compared to culture.

It is important to keep in mind that histopathological examination cannot be performed practically or ethically in live animals. Moreover, in this study histopathology did not detect any additional cases over and above those already

identified by culture, PCR or serology. Considering the time and skills required, inability to perform in a live animal and limited added value over the other tests, it is probably not one of the preferred samples for a practitioner to perform. In addition, the cost of the test (Table 1.1 - Chapter 1), if including the cost of the PME, is higher than PCR and Culture and the potential sensitivity, as reported in the literature, is relatively low. Although, sampling at slaughterhouse could be of use on a herd health basis in order to monitor Johne's disease on farm but this relies on adequate abattoir feedback to the farmer. This is the approach that takes place in Austria's slaughterhouses, where they check the carcasses in a similar way as tuberculosis is monitored in the UK (Flook, 2015).

Other authors have reported a higher sensitivity with tissue culture or tissue PCR compared to histopathology (Huda and Jensen, 2003; Sweeney *et al.*, 2006). The great advantage of this test is that it requires the same samples that are used for histopathology, but with a more valuable outcome. The sensitivity reported for tissue culture or PCR in some studies is even higher than faecal culture, the "gold standard" (Begg *et al.*, 2018). As a self-criticism exercise, tissue PCR could have been included in the study design, which could have helped to clarify some of the inconsistent results obtained. Although, formalin fixed samples have been stored and further tissue PCR could be carried out in the future. Several studies have demonstrated that formalin fixed samples and even paraffin-embedded samples can be used for tissue PCR (Plante *et al.*, 1996).

## 4.8 Testing in control schemes

Although various eradication schemes have specific rules and regulations to adhere to, Johne's disease control strategies should always be specific for each one of the farms that wish to reduce Johne's disease prevalence. This requires in depth knowledge of the farm management, farmer expectations and goals, knowledge of the laboratory tests available and expertise in interpretation of the results.

The use of the individual serology is part of many Johne's control schemes and is advised by many authors for identifying infected animals (Nielsen and Ersbøll, 2006). In high prevalence herds, a positive serology result will have a high

positive predictive value. Culling of the seropositive animals will tend to target MAP high-shedders and thus reduce the MAP infectious pressure on farm. If it is true that test and cull is important, the strategy of just culling seropositive animals has been shown to be insufficient in reducing Johne's disease prevalence. Test and cull should always be accompanied by hygiene improvement in the calving area, colostrum hygiene/management and internal biosecurity in order to break the faecal-oral route and avoid the infection of calves on farm. Prioritise culling of animals in farms with a high prevalence can be difficult as farmer might have to consider culling a large proportion of the herd. Such decisions can be made mainly based on production data. However, the serology percentage of positivity result could also be used to rank cull animals. This value will tend to target animals in a more advance stage of infection and probably the ones that will shed higher quantities of MAP. As shown by the results of this study, the correlation is not strong.

PCR and culture tests are a valuable tool, which is thought to be more sensitive than serology. The fact that animals tend to shed MAP before they show any antibody response is the main reason why PCR and culture have a  superior sensitivity when compared to serology. In the case where a negative serology result is followed by a positive result in either PCR/culture this could mean an incipient or early infection and this animal will probably be infected. However, if a positive result on serology is followed by a negative result on a PCR/culture test, it does not rule out the possibility that this animal is infected. The superiority of a PCR result over a serology result features in some control schemes (CHeCS for example). Given the high specificity of serology (99%) and the intermittent shedding of MAP described in previous research, it could be suggested inappropriate to rely on PCR/culture over the serology.

Literature review and results of this study have suggested that faecal culture and PCR can lead to false positive results due to a gastro-intestinal passage of MAP. As an example, if a farmer wants to buy a group of heifers:

- They should be sourced from a certified low risk farm if possible.

- PCR or culture would be more appropriate than serology (antibodies can fail to identify early stages of infection).

- This test should be done at the farm of origin and before they have been bought.

- A single positive result within that batch would indicate a Johne's positive farm and purchase of those animals would be not advised.

- At the individual level, the animal that yielded the positive result would probably be infected. However, there is a possibility that MAP found in the faeces could be due to a pass-through.

- A repeated PCR sample, a culture test or interpretation of the CT values could help to understand the MAP infectious status of that particular batch.

In beef farms, given the minimum handling compared to dairy cows and economic constraints, a yearly blood serology test is probably the preferred option. Calving hygiene management is generally difficult to control as 'snatch calving' is not really an option. The test and cull strategy is the most important measure in beef farms (Roussel, 2011). Although, other research has shown that test and cull alone does little to decrease prevalence of Johne's disease and does not decrease the costs associated to the disease in a 10-year period (Bennett *et al.*, 2010).

Control of Johne's disease requires a planned strategy that meets farmer's possibilities. Engagement of the farmer, appropriate testing strategy and avoiding new infections on farm are key to reduce prevalence of Johne's disease in any farm.

### 4.8.1 Sex and breed purpose

In the present study, female cattle made up the majority of the cases; 44 females and five (four castrated, one entire) males, which is unsurprising given the sex demographic in the Scottish cattle population, where 15% of cattle are male (Scottish Government, 2016). No tendency towards males or females could be found in this study, however exploring if sex was significant would require a larger sample size. No published literature was found assessing sex

predisposition in cattle. With regards to the purpose of the animal, an even distribution was found in beef and dairy, suggesting no difference between system. Some particular breeds were positive for culture in a larger percentage of the case population. All referred Jersey (6/6) and British/Belgian blue (4/4) were positive on faecal culture. Jerseys have been described as more susceptible breeds previously (Kirkpatrick and Shook, 2011). Although, no mention to British/Belgian blue breed was made in that paper or other papers consulted. Thus, a bigger sample size should be used if those issues need to be studied as relatively small numbers of these breeds were sampled in the current study.

## 4.9 History and farm of origin

Information about the animal history and farm of origin were gathered from the history forms taken prior to the animal referral. Herd size, biosecurity status, sheep co-grazing, previous history of the disease, Johne's health scheme membership, number of animals concurrently affected and onset of the clinical signs (in relation to the last calving) were compared to the culture result, with no significant relationship found in those factors. These data were gathered for completeness and not to study farm history data. A bigger population should be studied in order to assess risk factors. The only factors that were correlated to culture results were a Johne's test prior to admission and referral veterinary diagnosis.

One of the factors that is worth discussing is the onset of the clinical signs, which has been classically linked to stressful periods as calving. A two-month period from calving until the onset of the disease was chosen to study such relationship. Results from this study did not show any tendencies. It could be argued that two months might not be enough time to detect weight loss or diarrhoea on such animals and maybe a longer period would have been more appropriate to assess calving as a triggering factor. A three months period did not show any differences with the results presented on the results section above (data not shown).

The odds ratio (OR) of testing positive from an open herd compared to a closed herd was 0.6, although not significant, the tendency of these result indicated

that open herds were less likely to have culture positive animals. These results were unexpected as buying-in cattle is a known risk factor for Johne's disease (Rangel *et al.*, 2015). It could be hypothesized that farms experiencing Johne's disease could decide to become closed in order to avoid buying-in additional Johne's infected animals. The small number of animals referred from farms enrolled in Johne's health schemes could be explained either by the small number of commercial farms that are part of these schemes or by the lower prevalence of the disease on those farms. In addition, health scheme members, that have a good understanding of the disease may decide to cull animals when they test positive before clinical signs appear and when bovines have still a high carcase value. Further investigation should be carried out to explain both of these results. In addition, risk factor studies require a bigger sample size in order to correctly assess such factors.

The OR of testing positive when more animals are concurrently infected on farm was of four(p= 0.058). Since Johne's is an insidious disease, if one animal shows clinical signs, more than one animal is expected to be infected. It is not unexpected  that in culture positive animals, farmers reported that more than one animal was showing similar clinical signs at the same time on farm. If more animals were studied,  the results could show a higher significance.

Culture positive results were not significantly different regarding herd size. However, it could be argued that herd size categories should have been done differently for beef and dairy. Approximately 61.2% of the dairy farms in Scotland are smaller than 100 adult cows and 86.4% of the beef herds are smaller than 100 adult cows (ScottishGovernment, 2016). Thus, farm type and current national herd size data should be taken into account in future studies in order to assess this factor correctly.

The referral diagnosis listed corresponded with the main differential diagnosis that the referring veterinary reported at the time of referral. Thirteen animals were referred with a referral diagnosis of Johne's disease, which 12 were confirmed positive on culture. Nine animals had a referral diagnosis of Johne's disease without any laboratory test performed. Although, accuracy of those cases is of 88%, which is remarkable. These results are encouraging and suggest

that veterinarians in the field are gathering information (history of the animal, CE, expected prevalence on farm) to make an accurate presumptive diagnosis.

One animal was referred with a suspicion of Johne's disease from clinical exam but with no diagnostic testing performed prior to referral. On investigation, it was  negative on Johne's faecal culture. This cow was also negative on PCR and serology. This animal had no lesions suggestive of Johne's disease at the PME. However, this animal showed severe abomasitis caused by *Ostertagia* spp., which was proposed as the final diagnosis of this case. Cases like this, should encourage practitioners to perform a serology test on the animals that show clinical signs before jumping to a clinical diagnosis as not all thin and scouring adult cows have Johne's and they may be culled unnecessarily.

## 4.10 General limitations of the study

The cattle enrolled in this study were from a specific and limited section of the cattle population of Scotland. The animals came from a 221 Km radius of the Farm Animal Department of the University of Glasgow. In addition to the limited geographical area from which cases are donated, the animals referred to the hospital generally have little or no commercial value as they are received as donations for teaching. The donations also rely on farmer and veterinarian willingness to contact the Farm Animal Department, discuss the case and arrange transportation and licences (if required). All of these issues in combination meant that the study population was biased in terms of geographical area, commercial value, awareness of the teaching activities of the Farm Animal Hospital, co-operation of farmer/vet and fitness to transport. This is a relevant factor for the epidemiology and frequency of diseases that are diagnosed in this hospital where some chronic and incurable diseases, such as Johne's disease, are over-represented. Fitness for transport was assessed by a veterinarian on farm, which makes acute, severely diseased or lame animals less likely to arrive to the hospital. Thus, extrapolations of disease prevalence of the area cannot be made.

History gathering in this study was done in a retrospective manner. Information as gathered from the history forms from each individual case. These forms were completed during the phone call prior to case referral and information within

them relied on farmer's and Veterinarian's accuracy in the description of the history, farm characteristics and interpretation of terms such as open/closed. It also relied on the post-graduate student's accuracy and hand writing when completing the form. As a result, we cannot rule out any biased or slightly inaccurate information.

Other limitations of the study were the hospital space availability. On rare occasions, during peak times of case donation (spring and autumn) cases had to be prioritised; those collected were the ones with a highest teaching value within close proximity to the SVM. Other factors that may have hampered the collection of the cases were the location of the farm and transport availability.

The study design of the present work limited the sampling to animals with presenting signs that are typical of clinical Johne's disease and did not aim to look at animals in the subclinical stage on infection. A self-criticism could be made regarding the fact that most of the reported issues with diagnosing Johne's disease in cattle occur in subclinical stages of the disease. Thus, the relevance and impact of the results obtained has to be interpreted carefully and should be extrapolated to animals showing clinical signs, with caution in extrapolating the findings to the subclinical animal or herd screening type testing scenarios. On the other hand, the animals that showed poor BCS/weight loss and diarrhoea plus additional clinical signs could be considered as non-typical Johne's disease cases and even be seen as "control cases". Although, given that Johne's disease is known to predispose other diseases, such classification would be controversial and subjective in any future study design.

In this study the aim was not to gather a data from a large number of cases, but to get into the detail of each animal and try to understand and explain the result of each case. Although we acknowledge that a bigger sample size could have shown statistically significant differences in some of the factors studied, we did not aim to set up an epidemiological study and therefore did not attempt any power calculations to determine a sample size. Limitations described previously regarding case donation bias and limited time period for data collection were the major constraints. Using the model described, means that there would be scope to repeat the study over a longer period of time within the Farm Animal Hospital.

Test result data were missing in several cases, which has impaired a more powerful result analysis. The two main areas in which tests were missed were:

- Culture results: This was due to an initial misunderstanding with the reference laboratory, which just performed culture in cases where the PCR was negative, which resulted in a loss of 10 culture sample results.

- Histopathology samples: This was due to miscommunication. There were various staff members working in the Farm Animal Department and on occasions, post mortem requests for histopathology sample collection were not submitted. This resulted in a loss of 25 tissue samples.

However, the situation was amended as soon as possible once the mistakes were noticed. Improved communication and production/dissemination a set of clear protocols for the external laboratory, pathology department and Farm Animal department clinical staff would hopefully avoid this happening again.

Faecal culture was chosen as the gold-standard test and this is another limitation of the present study. As discussed before, faecal culture is not a perfect test. Due to the intermittent shedding pattern of the infected animals (mainly in a sub-clinical stage), the sensitivity of a single sample is low. Also, as discussed before, there is the theoretical possibility of false positive results due to a GIT faecal passage of MAP. Therefore, comparisons with other tests and Se and Sp values should be taken with caution. A Beyesian analysis could have been of value in order to compare test results between them and their performance. Bayesian studies are ideal, especially in studies where there is no perfect "gold standard". However, culture test has been chosen as the gold-standard in previous published studies and it is the assumed gold-standard for alive animals, this fact allows comparison of the results from the present study with results from previous literature.

## 4.11 Conclusions

This study was performed in animals showing clinical signs consistent with Johne's disease from different farms, production systems, breeds, sex and ages in Scotland that were submitted to a veterinary teaching hospital in west

Scotland. One finding of this study was the importance of a full clinical examination (CE) in cows showing clinical signs compatible with Johne's disease. Finding other clinical signs in addition to those characteristic of Johne's disease, lowered the probability of the animal to be suffering from Johne's disease. Thus, we would encourage practitioners to perform a full CE on animals showing clinical signs of Johne's disease in order to rule out other issues causing such clinical presentations. Such information will help the practitioner to make an immediate cost effective decision on Johne's disease testing, treatment of the animal or advise culling.

Another key finding of this paper was the good sensitivity (83%) and specificity (100%) of the serology test in cattle showing clinical signs. Results from this study suggest that this test should be always used as a first choice in animals showing clinical signs. Moreover, the very low probability of a false positive should make the practitioner confident with the serology result, even if the animal has a BCS over 2.5. This test would be very valuable in farms with a suspected high prevalence. In such cases, a positive result on serology would have a very high positive predictive value. In that case, false positive results due to pass-through, which could occur with PCR and culture tests, would be impossible with serology. In addition, the lower cost of the test compared to other available tests make serology an invaluable test, which should be used in control programs in farms with a suspected high prevalence of Johne's disease.

Additionally, this study found inconsistencies between PCR and culture results performed in the same faecal sample. Results from this study suggest that false positive results on PCR occur. It could be hypothesised that a highly contaminated environment could be the source of MAP genetic material on the faeces of the animals tested. Thus, this test would not be advised to be used to identify MAP-infected animals on an individual animal basis, and it would be discouraged in Johne's high prevalence farms.

This study also showed inconsistent results between culture results and the rest of the tests performed. The classical approach would be to classify such cases as MAP-infected animals. However, a comprehensive assessment of the information in this study can result in questioning of the "gold standard" status of the faecal culture test. A highly contaminated environment has the potential to cause pass-

through of the Johne's organism and thus yield false positive results on culture. This is a piece of evidence that adds to the studies that also suggest that false positive results from a culture test occur. As a result, a need for a definitive "gold-standard" method for Johne's disease in live animals would be needed, or, that it should be clearly reiterated to practitioners the limitations of the existing tests and their interpretation in the field. Moreover, as stated previously, the PCR test should be avoided in Johne's high prevalence farms and superiority of this test over serology should be made with caution.

Conclusions of this study highlight the importance of careful interpretation of the laboratory results by the veterinarians in the field in light of the clinical presentation of the animal. Current available tests give a piece of information about the Johne's disease infectious status of an individual animal. A holistic approach that takes into account the individual and farm history, full CE and critical interpretation of the laboratory results are needed in order to assign an infectious status of each animal regarding Johne's disease.

# Appendices

Appendix I: Veterinary history form and farmer herd and patient history forms.

| Date completed | VET PATIENT HISTORY | | | SCPAHFS Vet |
|---|---|---|---|---|
| Vet Name | | Phone number | | |
| Practice Name and Address | | | | |
| Farmer Name | | Farm name | | |
| Species | | Breed | | |
| Sex | | Age | | |
| Tag number | | | | |
| Initial complaint | | | | |
| Specific clinical signs | | | | |
| Diagnosis if reached | | | | |
| General Condition | | | | |
| Have any lab test been performed | Yes ☐   No ☐ | If yes give details | | |
| Date last seen by vet | /   / | Was it fit for transport | Yes ☐   No ☐ | |
| Was it previously examined by vet? | | Significant findings | | |
| Treatment given to animal | | | | |
| Response to treatment | | | | |
| How urgent is the case | ☐ High (collect within 4 days) | ☐ Medium (collect 5-10 days) | ☐ Low (Collect > 10 days) | |
| Is temperament suitable for a teaching facility | Yes ☐   No ☐ | | | |
| Does farmer have a herd or flock plan under regular review with practice | Yes ☐ No ☐ | Are there any ongoing herd/flock health issues (if yes please give details below) | Yes ☐   No ☐ | |
| Herd/flock health issues | | | | |
| Any other comments | | | | |

| Date Completed/reviewed | FARMER HERD/FLOCK | | SCPAHFS Vet |
|---|---|---|---|
| **General** | | | |
| Farmer Name | | | |
| Address | | | |
| Post Code | | Phone number | |
| Name of Vet | | Practice | |
| Farm CPH number | /    / | | |
| Farm type | Beef suckler commercial ☐   Beef suckler pedigree ☐  Beef fattening ☐<br><br>Dairy ☐ Heifer rearing ☐<br><br>Total number of BREEDING cattle on farm (total if fattening) _____<br><br>Sheep commercial ☐  Sheep pedigree ☐  Sheep fat lambs ☐<br><br>Total number of BREEDING sheep on farm _____<br><br>Other _____ | | |
| Other enterprises on Farm | Beef ☐   Dairy ☐  Sheep ☐  Pigs ☐  Poultry ☐  Other ☐ | | |
| Biosecurity status – Open = ANY animals bought in  (including bulls)<br><br>If open date animals last entered holding | Open ☐   Closed ☐<br><br><br>Date last animal entered        /    / | | |
| **Disease Status** | | | |
| BVD Disease status (cattle only) | Negative ☐          Not Negative ☐         Not known ☐ | | |
| BVD herd status confirmed on ScotEID (cattle only) | Yes ☐<br><br>No ☐ | BVD testing method (cattle only) | Tag testing ☐<br><br>Screen test ☐<br><br>Other ☐ |
| Health Scheme member, if yes what diseases accredited for | Yes ☐  No ☐   Diseases _____ | | |
| Other disease status (tick if present or previously diagnosed) | Johnes ☐     Lepto ☐    IBR ☐   OPA ☐  None ☐<br><br>Other diseases _____ | | |
| **Routine Treatments** | | | |
| Vaccines routinely used on farm | BVD ☐  Leptospirosis ☐  Rotavirus/Coronavirus ☐  Salmonella ☐<br><br>Clostrdial disease ☐    (please specify ) _____<br><br>Respiratory Disease ☐    (please specify ) _____<br><br>Other ☐   (please specify ) _____<br><br>No vaccine used ☐ | | |

| Endectocide routinely used on farm | Age Group of animal | Product used | When admin | |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

| **Mineral Supplementation** |||
|---|
| Do you use any mineral supplementation – if yes which method | Yes ☐ No ☐ <br><br> Bolus ☐ Lick ☐ Injection ☐ Drench ☐ Top dressing feeding ☐ |
| Is the farm known to be deficient in any minerals – If yes which mineral | Yes ☐ No ☐ <br><br> Copper ☐ Cobalt ☐ Selenium/Vit E ☐ Iodine ☐ Magnesium ☐ <br><br> Calcium ☐ Other _____ |
| Any Other significant information in relation to herd or flock | |

| Date completed | **FARMER Patient History** | | **SCPAHFS Vet** |
|---|---|---|---|
| **Farmer Name** | | **Farm Name** | |
| **Vet Name** | | **Practice Name** | |
| **Species** | | **Breed** | |
| **Sex** | | **Age** | |
| **Tag number** | | **Ear tags correct** | Yes ☐   No ☐ |
| **BVD antigen tested if 'not negative holding'** | Yes ☐<br>If yes, check antigen result on Scot EID website | No ☐<br>Animal needs antigen tested or BVD licence required before transport | |

| **Clinical** | |
|---|---|
| **Primary complaint** | |
| **Duration of primary complaint** | |
| **Other presenting signs** | |
| **Previous illness in this animal (if yes please give details)** | Yes ☐    No ☐ |
| **Treatment given to case** | |
| **Response to treatment** | |
| **only animal affected** | Yes ☐    No ☐<br><br>If no, number affected _____   Total in group _____ |
| **Is this animals temperament suitable for a teaching facility** | Yes         No ☐ |

| **Non clinical** | |
|---|---|
| **Purpose of animal on farm** | Milking Cow ☐   Suckler cow ☐   Fat ☐   Store Calf ☐<br><br>Replacement heifer ☐   Bull ☐   Growing bull ☐<br><br>Fat lamb ☐   Store lamb ☐   Breeding ewe ☐   Cull ewe ☐   Tup ☐<br><br>Other _____ |

| **BREEDING FEMALES ONLY** | | | |
|---|---|---|---|
| **Pregnancy status** | Pregnant ☐  Non pregnant ☐  Served not confirmed pregnant ☐ | | |
| **If pregnant due date if known** | /   / | **Date of last calving/lambing** | /   / |
| **Lactational status.** | Dry ☐   Milking ☐ | **If dry has dry cow therapy been administered?** | Yes ☐ No ☐ |

| Husbandry | | |
|---|---|---|
| **Where is the animal currently kept** | At grass ☐  <br><br>Date turned out  /  /  <br><br>Hill ☐  Upland ☐  Lowland ☐ | housed ☐ <br><br>Date housed  /  / |
| **Current feeding (tick more than one if req)** | Grass ☐  Grass silage ☐  Maize silage ☐  Hay ☐  <br><br>TMR ☐  In-parlour feeding ☐  Straw ☐  Concentrates ☐  <br><br>Mothers milk ☐  Artificial milk ☐  If milk Volume (litres) fed/day _____ | |
| **Any recent changes in feeding e.g. weaning (if yes date of change)** | Yes ☐  No ☐  Date  /  / | |
| Parasitology | | |
| **Date last treated for worms** | /  / | **Product Used** | |
| **Date last treated for fluke** | /  / | **Product Used** | |
| **Any other relevant information?** | | |

Appendix II: Consent form.

**University of Glasgow | School of Veterinary Medicine**

| DONATION OF FARM ANIMALS TO THE SCOTTISH CENTRE FOR PRODUCTION ANIMAL HEALTH & FOOD SAFETY, UNIVERSITY OF GLASGOW |
|---|

Dear Benefactor,

Thank you for donating your animal(s) to the Scottish Centre for Production Animal Health and Food Safety.  We are very grateful to you as this will enhance our students' exposure to clinical material. We have a responsibility to make you aware that any farm animals donated to us will not be returned to the farm of origin and that any clinical or other data gathered from the animal/s may be used for research purposes. If data from this case is used for research purposes it will be completely anonymised.

**Please complete the section below:-**

| Ear Tag | Species Cattle/Sheep | Breed | Sex M/F | DOB | Age |
|---|---|---|---|---|---|
|  |  |  |  |  |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |

As the owner/agent of the above animal/s I certify that I am donating them to Glasgow University. I understand that the animal/s will become property of Glasgow University and will not be returned. I also consent to data associated with the case being anonymised and potentially used for research purposes.

| Name of Benefactor |  |  |  |
|---|---|---|---|
| Address |  |  |  |
| Signature |  | Date |  |

*For donated teaching cases only, a fixed fee of £40 per Bovine and £20 per Ovine will be paid to the owner as a gesture of goodwill; this is because we are aware that taking a detailed history and arranging collection takes extra time. This donation is made on a per collection basis not on a per-animal basis, and is not intended to reflect the market value of the animal(s) donated.  Arrangements for invoicing must be made via telephone conversation prior to uplift of the animal(s); no payment will be made at the time of collection. Please provide an invoice on letter headed paper, or complete the attached invoice template at the time of collection.*

Appendix III: Clinical exam form.

University of Glasgow | School of Veterinary Medicine

**FARM ANIMAL CLINICAL EXAM FORM**

| OBSERVATIONS | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ear Tag | | | Breed | | | Weight | |
| Sex | Male | | Neutered Male | | Female | | Age |
| Demeanour | BAR | | | QAR | | Dull | |
| Gait | Normal | | | | Abnormal | | |
| | comments | | | | | | |
| Posture | Standing | | Sternal | | | Lateral | |
| Lameness Observed | Yes | No | | Comments | | | |
| Respiratory Rate | No of breaths/minute | | | | | | |
| Respiratory Character | Normal | | | Hypernoea | | Dyspnoea | |
| Rumination observed | Yes | No | | Comments | | | |
| Eating observed | Yes | No | | | | | |
| Coughing observed | Yes | No | | | | | |
| Diarrhoea observed | Yes | No | | | | | |
| Faecal staining observed | Yes | No | | | | | |
| Oedema Observed | Yes | No | | | | | |
| Skin/wool/hair | Normal | | | Alopecia | | | |
| | Ring worm | | | Lice | | | |
| Any other observation/comments | | | | | | | |

| BACK END | | | | | |
|---|---|---|---|---|---|
| Temperature °C | | | | | |
| Abdominal symmetry | Normal | | Left dorsal enlargement | | |
| | Right dorsal enlargement | | Left ventral enlargement | | |
| | Right ventral enlargement | | | | |
| Pulse character | Strong | | Thready | | |
| Pulse rate | beats/minute | | | | |
| Pulse regularity | Regular | Regularly irregular | | Irregularly irregular | |
| Vaginal MM | Normal | Cyanosed | | Pale | |
| | Congested | Icteric | | Petechiae | |
| Vulval discharge (without vaginal exam) | None detected | Mucus | | Fresh blood | |
| | Serohaemorrhagic | Purulent | | Mucopurulent | |
| | Brown/red | | | | |
| BCS | 1 | 2 | 3 | 4 | 5 |
| Urine stimulation | Normal | | Haematuria | | |
| | Pyuria | | Dilute | | |
| Lactation status | Lactating | | Dry | | |
| Udder and teats- Abnormalities | | | Comments | | |
| | LF Normal Abnormal | | | | |
| | RF Normal Abnormal | | | | |
| | LH Normal Abnormal | | | | |
| | RH Normal Abnormal | | | | |
| Penis | Normal Abnormal | | Comments | | |
| Prepuce | Normal Abnormal | | | | |
| Scrotum | Normal Abnormal | | | | |
| Testes | Normal Abnormal | | | | |
| Umbilicus | Normal | Purulent | Swelling | | |

| | Pain | | Heat | | Hernia | |
|---|---|---|---|---|---|---|
| Any other comments | | | | | | |

| HEAD | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Symmetry | Normal | | | | | Abnormal | | | | | |
| Muzzle | Wet | | Ulceration | | | Dry | | | Crusting | | |
| Nasal Discharge | None | | Purulent | | | Mucus | | | Expistaxis | | |
| Odour of breath | Normal | | Haliotosis | | | Ketotic | | | Ammonia | | |
| Submandibular LN | LEFT | Normal | | Enlarged | | RIGHT | Normal | | Enlarged | | |

| EYES | LEFT | | | | | RIGHT | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Vision | Normal | | Impaired | | | Normal | | Impaired | | | |
| | Blind | | | | | Blind | | | | | |
| Discharge | None | | Mucous | | | None | | Mucous | | | |
| | Purulent | | | | | Purulent | | | | | |
| Cornea | Normal | | Oedema | | | Normal | | Oedema | | | |
| | Ulceration | | Keratitis | | | Ulceration | | Keratitis | | | |
| Conjunctiva | Normal | | Pale | | | Normal | | Pale | | | |
| | Icteric | | Cyanosed | | | Icteric | | Cyanosed | | | |
| | Petechiae | | Chemosis | | | Petechiae | | Chemosis | | | |
| | Growth | | | | | Growth | | | | | |
| Eyelid | Normal | | Abnormal | | | Normal | | Abnormal | | | |
| Globe size | Normal | | Micropthalmia | | | Normal | | Micropthalmia | | | |
| | Enlarged | | | | | Enlarged | | | | | |
| Globe Position | Normal | | Stabismus | | | Normal | | Stabismus | | | |
| | Exophalmus | | Enophalmus | | | Exophalmus | | Enophalmus | | | |
| Globe Movement | Normal | | Nystagmus | | | Normal | | Nystagmus | | | |
| Pupil size | Normal | | Miosis | | | Normal | | Miosis | | | |
| | Dilated | | | | | Dilated | | | | | |
| PLR | Present | | Reduced | | | Present | | Reduced | | | |
| | Absent | | | | | Absent | | | | | |
| Menace | Present | | Reduced | | | Present | | Reduced | | | |
| | Absent | | | | | Absent | | | | | |

| Number of adult teeth | 0 | | | 2 | | 4 | | 6 | | 8 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Broken mouth | Yes | | | | | No | | | | | |
| Gag exam performed | Yes | | | | | No | | | | | |
| Tongue | Normal | | Swelling | | Ulceration | | Abnormal tone | | | | |
| Hard palpate | Normal | | Swelling | | Ulceration | | Cleft palate | | | | |
| Larynx auscultation | Normal | | | | Harsh | | | | | | |
| Trachea auscultation | Normal | | | | Harsh | | | | | | |
| Tracheal pinch test | Cough | | | | No cough | | | | | | |
| Jugular distention | LEFT | Yes | | No | | RIGHT | Yes | | No | | |
| Jugular reflux test | LEFT | Normal | | Abnormal | | RIGHT | Normal | | Abnormal | | |
| CRT gums | Numerical field secs | | | | | | | | | | |
| Gum palpitation | Normal | | | Tacky | | Dry | | | | | |
| Skin tent | 1 | | 2 | | 3 | 4 | | 5 | | >5 | |
| % Dehydration | | | | | | | | | | | |
| Any other comments | | | | | | | | | | | |

| LEFT SIDE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Heart rate (beats min) | | | | | | | | |
| Heart Auscultation | Normal | | Muffled | | | Murmur | | |
| Heart rhythm | Regular | | Regularly | | | Irregularly irregular | | |
| Lung auscultation | Normal | | | | Wheeze | | | |
| | Crackles | | | | No sound | | | |
| Lung percussion | Normal | | | | Pain | | | |
| | Heart Enlarged/ | | | | Decreased resonance | | | |
| Withers test | Positive | | | | Negative | | | |
| Rumen auscultation | Number secs between ruminal contractions | | | | | | | |
| Rumen palpitation for layers | Normal | | | | Abnormal | | | |
| Percussion auscultation LDA | Absent | | | | Present | | | |
| If ping splash on Ballottement | Absent | | | | Present | | | |
| Pre-femoral lymph node | Normal | | | | Enlarged | | | |
| Pre-scapular lymph node | Normal | | | | Enlarged | | | |

| | | LEFT FORE | | | | LEFT HIND | | | |
|---|---|---|---|---|---|---|---|---|---|
| Feet | Normal | | Overgrown | | Normal | | | overgrown | |
| | Swollen coronary band | | Other | | Swollen coronary band | | | Other | |
| Joints | Normal | | Hot | | Normal | | | Hot | |
| | Swollen | | Painful | | Swollen | | | Painful | |
| | Deformed | | | | Deformed | | | | |
| Limbs | Normal | | Swollen | | Normal | | | swollen | |
| | Deformed | | | | Deformed | | | | |
| Foot lifted | Yes | | | | No | | | | |

| RIGHT SIDE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Heart auscultation | Normal | | Muffled | | | Murmur | | |
| Heart rhythm | Regular | | Regularly | | | Irregularly irregular | | |
| Lung auscultation | Normal | | | | Wheeze | | | |
| | Crackles | | | | No sound | | | |
| Lung percussion | Normal | | | | Pain | | | |
| | Heart enlarged | | | | Decreased resonance | | | |
| Percussion auscultation RDA | Absent | | | | Present | | | |
| If ping splash on Ballottement | Absent | | | | Present | | | |
| Pre-femoral lymph node | Normal | | | | Enlarged | | | |
| Pre-scapular lymph node | Normal | | | | Enlarged | | | |
| Liver enlargement | Present | | | | Absent | | | |

| | | RIGHT FORE | | | | RIGHT HIND | | | |
|---|---|---|---|---|---|---|---|---|---|
| Feet | Normal | | Overgrown | | Normal | | | overgrown | |
| | Swollen coronary band | | Other | | Swollen coronary band | | | Other | |
| Joints | Normal | | Hot | | Normal | | | Hot | |
| | Swollen | | Painful | | Swollen | | | Painful | |
| | Deformed | | | | Deformed | | | | |
| Limbs | Normal | | Swollen | | Normal | | | swollen | |
| | Deformed | | | | Deformed | | | | |
| Foot lifted | Yes | | | | No | | | | |
| Left Side/Right side comments | | | | | | | | |

| RECTAL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Evidence of Peritonitis** | Absent | | | Local | | | | General | |
| | Comments | | | | | | | | |
| Rumen | Normal | | | Gassy | | Empty | | Firm | |
| Kidney | Normal | | | Enlarged | | | | Painful | |
| Caecum | Not palpated | | | | | Enlarged | | | |
| Intestine | Not palpated | | | | | Enlarged | | | |
| Bladder | Not palpated | | | Palpated normal | | | Palpated enlarged | | |
| Uterus | Not pregnant | | | Pregnant | | | Enlarged (not pregnant) | | |
| | Comments | | | | | | | | |
| Ovaries | Right | Small | | Multiple small follicle | | | One large follicle | | CL | |
| | Left | Small | | Multiple small follicle | | | One large follicle | | CL | |
| Faeces | Normal | | | Diarrhoea | | Melena | | Dry | |
| | None Present | | | Haemorrhagic | | Mucoid | | | |
| Rectal ultrasound exam performed | | | Yes | | | | No | | |

| VAGINAL EXAM | | | | | | |
|---|---|---|---|---|---|---|
| Cervix | Open (>2 fingers) | | | Closed (<2 fingers) | | |
| Vagina | Normal | | | Tear | | |
| | Bruise | | | Polyp | | |
| Vulva | Normal | | | Swollen | | |
| | Tear | | | Poor confirmation | | |
| Vaginal discharge appearance | None detected | | Mucus | | Fresh blood | |
| | Mucopurulent | | Purulent | | Brown | |
| Vaginal smell | Present | | | Absent | | |
| Comments | | | | | | |

| SUMMARY | |
|---|---|
| **Problem List** | |
| **Differential Diagnosis** | |
| **Plan** | |

Appendix IV: Haematology and biochemistry results.

**CLINICAL PATHOLOGY (RE-PRINT)**

Veterinary Diagnostic Services, School of Veterinary Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK
Tel: +44 (0)141 330 5777 Fax: +44 (0)141 330 5748
email: vet-sch-vds@glasgow.ac.uk  Website: www.glasgow.ac.uk/vds
University of Glasgow, charity number SC004401

**University of Glasgow**
**School of Veterinary Medicine**

**Arlene Macrae**
**FARM ANIMAL MEDICINE &**
**TEACHING(134760-01)\*\*\*\***
**University of Glasgow**
**Veterinary School**
**Bearsden**
**GLASGOW**

Your Ref

Owner
Animal
Species
Breed
Age
Sex

159

| Sample No | | Sent | |
| Sample | EDTA Hep | Received | |
| Reason | | Report Date | |

| Test | Result | Unit | Reference Range | |
| --- | --- | --- | --- | --- |
| 023 - Basic Ruminant | | | | 19.00 |
| Urea | 2.1 | mmol/L | - 8.3 | |
| Creatinine | 65 | umol/L | 53 - 132 | |
| GGT | 86 | U/L | - 27 | |
| Total Protein | 76 | g/L | 52 - 84 | |
| Albumin | 32 | g/L | 21 - 34 | |
| Globulin | 44 | g/l | 29 - 56 | |
| Albumin:Globulin Ratio | 0.73 | | | |
| GLDH | 75.7 | U/L | - 10 | |
| 005 - Haem - Full w.profile | | | | 17.50 |
| RBC | 4.7 | x10 E12/l | 5.0 - 10.0 | |
| Hb | 8.4 | g/dl | 8.0 - 15.0 | |
| HCT | 25.8 | % | 24 - 46 | |
| MCV | 54.9 | fl | 40.0 - 60.0 | |
| MCH | 17.8 | pg | 11.0 - 17.0 | |
| MCHC | 32.5 | g/dl | 30.0 - 36.0 | |
| RDW | 16.5 | % | | |
| WBC | 15.08 | x10 E9/l | 4.0 - 12.0 | |
| Neutrophils | 13.421 | x10 E9/l | 0.6 - 4.12 | |
| Lymphocytes | 1.508 | x10 E9/l | 2.5 - 7.5 | |
| Monocytes | 0.151 | x10 E9/l | 0.025 - 0.84 | |
| Eosinophils | 0 | x10 E9/l | 0.00 - 2.40 | |
| Basophils | 0 | x10 E9/l | | |
| PLT | 404 | x10 E9/l | 100 - 800 | |
| MPV | 7.5 | fl | | |
| PCT | 0.3 | % | | |
| PDW | 55.1 | % | | |

Haematology Smear Report

The red cells show mild spiculing. The neutrophils are poorly preserved but suspect a mild left shift with slight toxic change. RB

END OF REPORT                                    Total Cost exc. VAT      £36.50

## Appendix V: Johne's laboratory tests submission form, SRUC.

| Your ref | | Owner | |
|---|---|---|---|
| Date of despatch | | Clinical notes | |
| Sample | | Examination requested | |

---

**FARM ANIMAL SUBMISSION FORM**
**Veterinary Services**

**SAC CONSULTING**

### CLIENT'S NAME AND ADDRESS

| CPHH No | / / | Postcode | |
|---|---|---|---|

**For Lab use only**

| Date received | | Lab ref | |
|---|---|---|---|
| Suites/DETS | | AA Code | |
| | | Cross Ref | |
| | | Duty Vet | |

### ADDRESS WHERE ANIMAL IS KEPT, IF DIFFERENT FROM ABOVE

| CPHH No | / / | Postcode | |
|---|---|---|---|

**SPECIMENS**

| Specimen type | | | |
|---|---|---|---|
| No. of specimens | | No. of animals sampled | |
| Date collected | | Date sent | |
| Crofter? | YES / NO | Practice Ref | |
| Is this the first specimen from this case/outbreak | | | YES / NO |
| Previous lab Ref. No | | | |

### VETERINARY PRACTICE NAME AND ADDRESS

| Clinician | | Postcode | |
|---|---|---|---|
| Invoice to: | | PO no: | |

### ANIMAL DETAILS

| ANIMAL DETAILS | | Species | | | | Breed | |
|---|---|---|---|---|---|---|---|
| Sex | | Male | | Female | | Castrate | Mixed |
| Age (State number) | | | | Days / weeks / months / years / mixed (Indicate as appropriate) | | | |
| | | Adult | | Unknown | | Stillborn-foetus | Weaned YES/NO |
| Total Stock at risk | | No. In Affected Group | | No. Affected | | No. Dead | |

### FARM TYPE  —  Please complete all sections  —  Housing

| Cattle | Sheep | Pig | Other Classes | | Avian | Housed |
|---|---|---|---|---|---|---|
| Calf Rearer | Hill (extensive) | Breeding | Dairy (small ruminant) | Captive/zoo | Breeder/parent | Outdoors |
| Beef finisher | Lowland | Rearing | Fibre | Wild | Production | Mixed |
| Dairy | Lamb finisher | Finishing | Other Farmed (Rabbit, fish, deer) | Pet | Game | Other |
| Suckler | ORGANIC: YES/NO | | | Unknown | | Unknown |

### MAIN CLINICAL SIGN

| Healthy | Eye disease | Malaise (Live animal) | Recumbent | Other |
|---|---|---|---|---|
| Abortion | Found dead | Mastitis (Clinical) | Respiratory | Specify |
| Repro-not abort | Wasting / poor condition | Mastitis (Subclinical) | Skin | |
| Diarrhoea | Lameness | Milk drop | Unknown | |
| GIT not Diarrhoea | Musculoskeletal-not lame | Nervous signs | Urinary | Not Applicable |

### REASON FOR SUBMISSION

| Diagnostic | Follow up prev. sub | Monitoring | Other | Project | QA | Screen for Notif Dis |
|---|---|---|---|---|---|---|

Please tick box if sample cannot be used for anonymous surveillance ☐

| Clinical history: clinical signs, diet, treatment, vaccinations etc. | Animal I. D. | Sample I. D. |
|---|---|---|
| | 1 | |
| | 2 | |
| | 3 | |
| | 4 | |
| | 5 | |
| | 6 | |
| | 7 | |
| | 8 | |

*Please turn over for tests required section*

**TEST REQUIRED (Tick boxes below or write here. For full range of tests see current price list).**

|  |
|---|
|  |

| MICROBIOLOGY |
|---|
| Johne's Microscopy |
| Bacterial Culture & sens |
| Salmonella Screen |
| **PORCINE PCR TESTS** |
| M hyopneumoniae |
| Brachyspira spp |
| L intracellularis |
| PRRS virus |
| Atrophic rhinitis |

| HAEMATOLOGY |
|---|
| Full Haematology |
| RBC, WBC, PCV, Hb |
| WBC & Diff |

| SEROLOGY | |
|---|---|
| BVD Ab | BVD Ab & Ag |
| BVD Ab & Ag if Ab low or negative | |
| IBR | L hardjo |
| Johnes | N caninum |
| Liver Fluke | |

| PARASITOLOGY |
|---|
| Worm egg/Cocci Oocyst Count |
| Liver Fluke |
| Lungworm Larvae |
| Comprehensive (all above) |
| Worm scan (10 samples required) |
| Bulk Fluke (10 samples required) |

| HISTOPATHOLOGY | |
|---|---|
|  |  |

| SKINS | |
|---|---|
| Microscopic | Comprehensive |
| Micro & Ringworm culture | |

| BIOCHEMISTRY | | | | |
|---|---|---|---|---|
| Calcium | Magnesium | Phosphorous | ZST | |
| Cu | GSHPx | B12 | Vit E | Vit A |
| GLDH | GGT | AST | CK | |
| Total Protein | Albumin | Globulin | Urea | |
| Butyrate | Glucose | NEFA | Pepsinogen | Iodine |

**PACKAGE REQUIRED (Tick as required)**

| BIOCHEMISTRY |
|---|
| Ruminant Trace Element |
| Ruminant III Thrift |
| Myopathy |
| Ruminant Mineral Status |
| Downer Cow Profile |
| Fatty Liver |
| Ruminant Energy/Protein Status |
| Ewe Metabolic Disease Profile |
| Bovine Mini Metabolic Profile |
| Individual Clinical Profile |

| ENTERITIS | | |
|---|---|---|
| Neonatal | | Basic enteritis |
| Young ruminant | | |
| Adult ruminant | | |
| Weaned pig | 1 | 2 |
| Grower/finisher pig | | |

| REPRODUCTIVE FAILURE | | |
|---|---|---|
| L.hardjo, BVD, N.caninum, IBR* | | |
| EAE, Toxoplasma | | |
| Porcine | (a) | (b) |

| RESPIRATORY DISEASES |
|---|
| FAT for IBR, RSV, PI3* |
| FAT as above & Bacteriology |
| Virus isolation on FAT Negative |
| Serology for RSV, PI3, IBR, BVD, H.somnus, M.bovis* Single/Paired* |
| Porcine Respiratory Serology |

| MASTITIS |
|---|
| Mastitis package (bacteriology & sens) |
| Mastitis – bacteriology only |
| Cell count |

| |
|---|
| * delete as necessary |

Appendix VII: Individual case TPR form.

**SCOTTISH CENTRE FOR PRODUCTION ANIMAL HEALTH AND FOOD SAFETY**
University Of Glasgow School of Veterinary Medicine
Bearsden Road, Glasgow G61 1QH

```
┌─────────────────────┐
│                     │
│                     │
│     Case Label      │
└─────────────────────┘
```

## PROGRESS / TREATMENT RECORD

| Date & Time | Clinical Findings | Progress / Diagnostic testing undertaken / Treatment & Comments | Clinician |
|---|---|---|---|
| | T<br>P<br>R | Biochem ☐   Haem ☐   Urinalysis ☐<br><br>Faecal samples: Bacto ☐ Parasitology ☐<br><br>Milk samples bacto ☐ Serology ☐<br><br>Radiography ☐<br><br>Other ☐<br>............................................<br>............................................ | |
| | | **Drugs used & amount** / **Batch No.** | |

| Date & Time | Clinical Findings | Progress / Diagnostic testing undertaken / Treatment & Comments | Clinician |
|---|---|---|---|
| | T<br>P<br>R | Biochem ☐   Haem ☐   Urinalysis ☐<br><br>Faecal samples: Bacto ☐ Parasitology ☐<br><br>Milk samples bacto ☐ Serology ☐<br><br>Radiography ☐<br><br>Other ☐<br>............................................<br>............................................ | |
| | | **Drugs used & amount** / **Batch No.** | |

PAGE............

Appendix VIII: Post-mortem individual case report.

Sample No ▮▮▮▮▮▮

# University of Glasgow

School of Veterinary Medicine

**PATHOLOGY REPORT**
**Postmortem Examination**

Veterinary Diagnostic Services, School of Veterinary Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK
Tel: +44 (0)141 330 5777 Fax: +44 (0)141 330 5748
email: vet-sch-vds@glasgow.ac.uk   Website: www.glasgow.ac.uk/vds
University of Glasgow, charity number SC004401

| | |
|---|---|
| Pamela Johnston<br>ANATOMICAL PATHOLOGY (<br>118552-01 )<br>Jarret Building<br>GUVS | Your Ref<br>Owner ▮▮▮▮▮▮<br>Animal ▮▮▮▮▮▮ ▮▮▮▮▮▮<br>Species CATTLE<br>Breed ▮▮▮▮▮▮<br>Age ▮▮▮▮▮▮<br>Sex ▮▮▮ |

| Sample No ▮▮▮▮ | | Sent ▮▮▮▮ |
|---|---|---|
| Clinical Diag | Johne's, fluke, pne | Received ▮▮▮▮ |
| Slides | | Report Date ▮▮▮▮ |

**Clinical History**
Admitted to Galloway ▮▮▮▮ BCS 0.5. Extremely necrotic foetid breath, superlative nasal discharge. Loose faeces. Jugular pulses. Reported Hx of heart murmur. Possibly related to cachexia. Sub mandibular mass. Samples taken for Johne' s and fluke. Euthanized on welfare grounds

**Gross Pathology**
Submitted for a teaching post-mortem examination is a ▮▮▮▮▮▮▮▮▮▮ cow in poor body condition and good post-mortem condition (the animal has been dead for less than 6 hours). Ear tag: ▮▮▮▮▮▮

The mucosae and the sclera are moderately pale.
The fascia of the mylohyoid muscle has a gelatinous consistency and is wet (oedema).
The lungs have a diffuse pale discolouration. In the pericardial sac there are small (less than 300ml) amounts of a clear yellow tinged fluid (effusion).
The left lobe of the liver is slightly reduced in size (atrophy) and has multifocal white and firm areas, especially at the lateral edge (fibrosis). Numerous variably-sized (ranging from 0.5 x 2 cm to 0.5x 5 cm) white and firm tracts are also observed in the visceral surface of this lobe. On cut surface these correspond to bile ducts with a moderately thickened white wall (fibrosis). In the lumen of some bile ducts as well as in the the the gall bladder there are numerous adult trematode parasites (*F. hepatica*). The right lobe is slightly enlarged (hypertrophy). Hepatic lymph nodes are markedly enlarged and wet on cut surface.
The wall of the ileum is diffusely and markedly thickened. When dissected, the mucosa has a granular appearance and forms thick irregular folds (corrugation). Numerous lymphatic vessels are distended on the serosal surface of the ileum and in the mesentery. Mesenteric lymph nodes are moderately enlarged.

**Morphological Diagnosis**
Pericardial sac: Effusion, serous, mild.
Liver: Pericholangitis, multifocal, marked, chronic with left liver lobe atrophy and fibrosis, marked and right lobe hypertrophy, mild.
Hepatic lymph node: Lymphadenopathy, marked.
Ileum: Enteritis, granulomatous, diffuse, marked, chronic with lymphangitis, multifocal

**Comment**

# University of Glasgow

School of Veterinary Medicine

**PATHOLOGY REPORT**
**Postmortem Examination**

Comments

The most relevant macroscopic findings in this animal are the chronic granulomatous enteritis, lymphangitis and lymphadenitis, likely associated with the infection with *M. avium subsp paratuberculosis*, and the marked pericholangitis, fibrosis and atrophy of the left hepatic lobe caused by *F. hepatica*.

The presence of oedema in the intermandibular region and the pericardial serous effusion are likely related to protein losing enteropathy.

The pale mucosae, sclera and lungs are indicative of anaemia which was likely caused by the chronic disease status.

This is a final gross report.

, DVM, PhD, MRCVS
Anatomic Pathology Resident

Dr
**BVM&S PhD PGCHE FHEA MRCVS**

| | |
|---|---|
| Cost | £ 370.00 |
| VAT | £ 0.00 |
| **Total** | **£ 370.00** |

Appendix IX: Total number of animal referred during the duration of the study shown by breed.
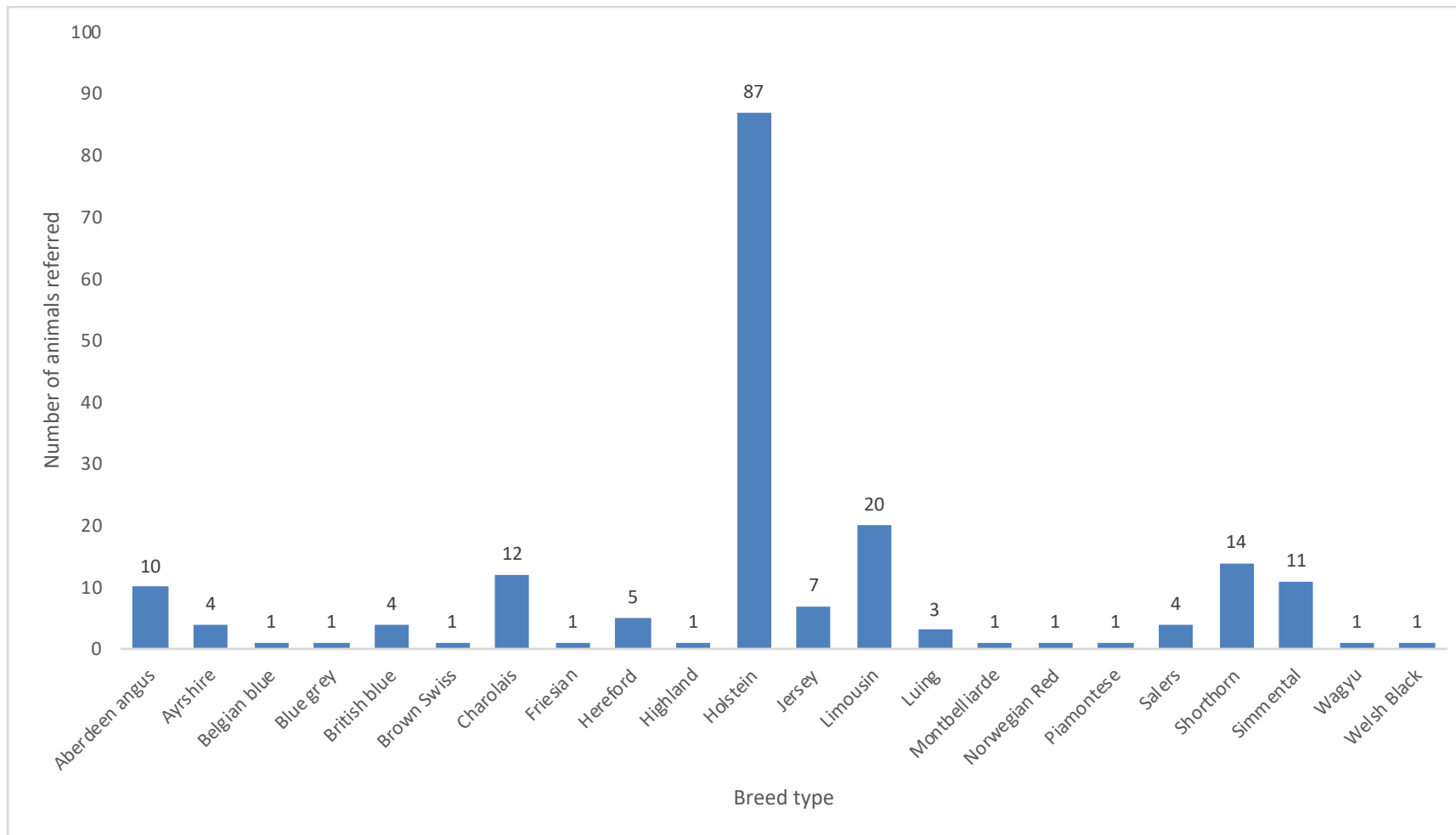
Appendix X: Summary of the findings and laboratory results regarding Johne's disease.

| Case No. | Age in months | Clinical signs | PCR | Culture | Serology | Gross path | Histology |
|---|---|---|---|---|---|---|---|
| 259760 | 22 | 1 | + | - | - | - | NS |
| 259835 | 55 | 1 | + | + | + | + | NS |
| 259971 | 27 | 1 | + | NS | + | + | NS |
| 259990 | 32 | 1 | - | NS | - | NS | NS |
| 260138 | 32 | 1 | + | + | + | + | NS |
| 260267 | 123 | 2 | NS | NS | - | - | NS |
| 260366 | 60 | 1 | + | NS | + | + | NS |
| 260411 | 21 | 1 | + | NS | + | + | NS |
| 260412 | 17 | 1 | + | NS | + | + | NS |
| 260665 | 44 | 1 | - | NS | - | - | NS |
| 260780 | 70 | 1 | + | + | + | + | + |
| 261324 | 60 | 2 | - | + | + | - | - |
| 261325 | 44 | 1 | - | + | - | - | - |
| 261326 | 59 | 1 | + | + | + | + | + |
| 261549 | 73 | 1 | NS | NS | + | + | NS |
| 261933 | 21 | 1 | - | - | - | - | NS |
| 261985 | 105 | 1 | + | + | + | + | + |
| 262116 | 13 | 2 | - | NS | NS | + | NS |
| 262366 | 24 | 2 | - | - | - | - | NS |
| 262647 | 9 | 2 | - | + | - | - | - |
| 262832 | 182 | 2 | - | - | - | NS | NS |
| 262850 | 12 | 2 | NS | NS | - | - | NS |

| 263084 | 53 | 2 | + | + | + | + | + |
| 263085 | 86 | 2 | - | - | - | - | - |
| 263444 | 34 | 1 | + | + | + | + | + |
| 263555 | 51 | 1 | + | + | + | - | + |
| 263611 | 32 | 2 | - | - | - | - | NS |
| 263612 | 16 | 2 | - | - | - | - | NS |
| 263971 | 48 | 1 | + | + | + | + | + |
| 263984 | 59 | 2 | - | - | - | - | NS |
| 263991 | 64 | 2 | - | - | - | - | - |
| 264008 | 26 | 2 | - | - | - | - | NS |
| 264033 | 56 | 2 | + | + | + | + | + |
| 264041 | 35 | 2 | - | - | - | - | NS |
| 264045 | 14 | 2 | + | - | - | - | - |
| 264048 | 68 | 2 | - | - | - | - | - |
| 264054 | 60 | 1 | + | + | + | + | + |
| 264065 | 64 | 1 | + | + | + | + | + |
| 264075 | 92 | 2 | - | - | - | - | NS |
| 264149 | 70 | 1 | + | + | + | - | + |
| 264150 | 81 | 1 | + | + | + | + | + |
| 264160 | 141 | 2 | + | - | - | - | - |
| 264161 | 110 | 1 | + | + | + | + | + |
| 264162 | 59 | 2 | - | - | - | NS | NS |
| 264165 | 34 | 1 | + | + | + | + | + |
| 264177 | 16 | 2 | - | - | - | - | - |
| 264178 | 25 | 2 | - | - | - | - | - |

| 264215 | 10 | 1 | - | + | - | - | NS |
|---|---|---|---|---|---|---|---|
| 264238 | 50 | 1 | + | + | + | + | + |
| 264241 | 10 | 1 | + | - | - | + | - |
| 264245 | 53 | 2 | + | + | - | NS | NS |
| 264267 | 62 | 1 | - | - | - | - | - |
| 264283 | 105 | 2 | - | - | - | - | - |
| 264284 | 85 | 2 | - | - | - | - | NS |
| 264299 | 70 | 1 | + | + | + | + | + |
| 264310 | 62 | 1 | + | + | + | + | + |
| 264348 | 96 | 2 | - | - | - | - | - |
| 264350 | 46 | 2 | - | - | - | - | - |
| 264354 | 121 | 2 | - | - | - | - | - |
| Total+ | | 30 | 28 | 24 | 25 | 24 | 17 |
| Total- | | 29 | 28 | 25 | 33 | 31 | 16 |
| Total | | 59 | 56 | 49 | 58 | 55 | 33 |

(+) positive result, (-) negative result, (NS) not sampled

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