
Associations between Johne's Disease and fertility in UK dairy herds

Thesis submitted for Masters of Veterinary Medicine

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Abstract

The objective of this observational study was to quantify the association between *Mycobacterium avium* subspecies *paratuberculosis* (MAP) antibody status and risk of insemination and subsequent conception, in UK dairy cattle. Longitudinal milk recording, fertility and MAP antibody enzyme-linked immunosorbent assay (ELISA) milk test data were collated retrospectively from 121,762 lactations in 78 herds. Datasets were structured into appropriate units to suit outcomes and enable temporal association between current and future MAP status, and fertility measures. Current MAP status was categorised according to most recent status within 180 days, with time-related future MAP status assigned based on MAP antibody ELISA milk test data for each cow. Multilevel multivariable logistic regression models were used to evaluate associations between MAP status and 21-day pregnancy and submission rate and conception risk. Posterior predictions and cross-validation techniques were used to assess model fit and check model building assumptions.

A negative association was found between risk of insemination (Odds Ratio [OR], 0.78; 95% Credible Interval [CI], 0.66 - 0.92) and conception occurring (OR, 0.65; CI, 0.50 - 0.84) and transition from negative to non-negative MAP test status in the next 30 to 90 days. A positive association was observed between risk of insemination (OR, 1.34; CI, 1.16 - 1.52) and conception occurring (OR, 1.26; CI, 1.11 - 1.43) and transition from negative to non-negative MAP test status in the next 90 to 180 days. Current positive MAP test status was negatively associated with insemination risk (OR, 0.59; CI, 0.49 - 0.70) and positively associated with conception risk (OR, 1.12; CI, 0.96 - 1.30). Herd managers will have had access to test results, declaring cows with past recent or multiple positive MAP antibody ELISA results not to be bred, negatively influencing insemination risk. Overall, these results reveal complex temporal associations between a positive MAP antibody ELISA result and dairy cow fertility outcomes. This was identified particularly prior to a positive MAP antibody ELISA result, with risk of both insemination and conception increasing then decreasing before a non-negative MAP test status.

Acknowledgements and declarations

A summary of the project discussed in this thesis is published as Reynolds et al. (2023), “Associations between Johne’s Disease and fertility in UK dairy herds”. *The Veterinary Journal*; DOI: **10.1016/j.tvjl.2023.106015**.

A review of the data quality screening measures developed during this project is also published in Reynolds J., Hudson C. (2023). “Herd health: approach to assessing fertility data quality”. *In Practice*; 45(4); DOI:**10.1002/inpr.311**

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Glossary

AUROC: Area under the receiver operating characteristic curve

CFU: Colony Forming Units

CL: Corpus Luteum

Ct: Cycle threshold

DA: Displaced abomasum

DIM: Days In Milk

DNA: Deoxyribonucleic acid

ECBHM: European College of Bovine Health Management

ELISA: Enzyme-linked immunosorbent assay

EU: European Union

HACCP: Hazard Analysis and Critical Control Point

IgG1: Immunoglobulin G1

IL: Interleukin

MAP: *Mycobacterium* subspecies *avium paratuberculosis*

MCMC: Markov chain Monte Carlo

MiRNA: Micro ribonucleic acid

mRNA: Messenger ribonucleic acid

PCR: Polymerase Chain Reaction

PPD: Purified protein derivative

QTL: Quantitative trait locus

R₀: Reproduction number

RAMP: Risk assessment and management plan

RCVS: Royal College of Veterinary Surgeons

s/p ratio: Sample to positive ratio

SCC: Somatic cell count

SICCT: Single Intradermal Comparative Cervical Tuberculin Test

T_H1/2: T-helper cell type 1/2

TLR: Toll Like Receptor

TNF- α : Tumour Necrosis Factor Alpha

WOAH: World Organisation for Animal Health

Chapter 1: Introduction and Literature review

1.1 Context

This thesis has been completed as part of the Residency and Masters Programme in Dairy Herd Health and Production at the School of Veterinary Medicine and Science, University of Nottingham. The programme consists of a combination of clinical, research and teaching activity designed to prepare candidates for admission to the ECBHM examination and subsequent qualification as a European Veterinary Specialist. Details of the authors fulfilment of the ECBHM standard residency requirements and examination are included in Appendix 1 and 2, with the certificates for recognition of European Veterinary Specialist and RCVS Specialist statuses included in Appendix 3 and 4.

1.2 Bacterial characteristics

Paratuberculosis (Johne's disease) is a fatal, transmissible and chronic condition of both ruminants and non-ruminants, characterised by inflammatory granulomatous enteritis and caused by Gram-positive *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Sweeney, 2011). MAP is an acid-fast obligate intracellular rod shaped organism, which requires mycobactin for its slow growth (Harris and Barletta, 2001) and was identified first in 1912 (Twort and Ingram, 1912). The bacterium has fastidious growth requirements with a minimum generation time of 20 hours and an 8 to 12 week period before visible growth (Rowe and Grant, 2006). MAP is a non-spore forming, non-motile organism, with the high cell wall lipid concentration implicated in its virulence and success of intra-cellular survival (Brennan, 1989). Lipoarabinomannan is present in all cell wall layers and inhibits macrophage activation and T-cell stimulation, enhances TNF- α production and removes cytotoxic substances from the cytosol (Barrow, 1997).

MAP is classified as a slow growing *Mycobacteria* (Veyrier *et al.*, 2009) (Figure 1); Type S (sheep-type) and Type C (cattle-type) are the main strains recognised, with Type S isolated in mostly sheep and experimentally in cattle and Type C without host preference, present in cattle, sheep, goats, deer, bison, buffalo and humans (Bryant *et al.*, 2016), with increasing evidence of interspecies transmission, especially in co-grazed animals (Stevenson, 2015). Type B strain has been identified as a sub lineage of Type C strains (Bryant *et al.*, 2016) and demonstrated to infect domestic, wildlife species and humans (Kumar *et al.*, 2008).

Three subtypes have been delineated, Types 1 to 3, although association with disease severity is unclear (Figure 2) (Stevenson, 2015); Type 1 is associated with the Type S 'pigmented ovine' strain, Type 2 associated with the Type C strains (Stevenson *et al.*, 2002) and Type 3 associated with the Type S group (Stevenson, 2015).

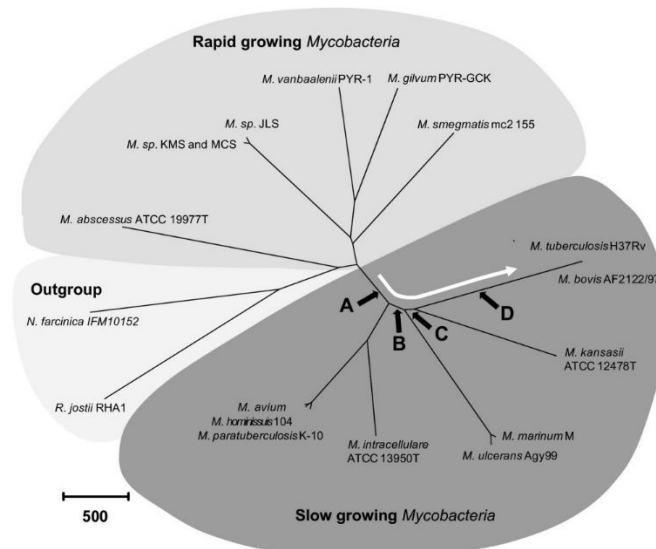


Figure 1: *Mycobacterium* genus phylogeny, with slow and fast-growing lineages represented. A to D represent categories defined by different gene acquisitions by horizontal gene transfer (Veyrier *et al.*, 2009; copyright Frederic Veyrier. Reproduced under the Creative Commons Licence from BMC Evolutionary Biology).

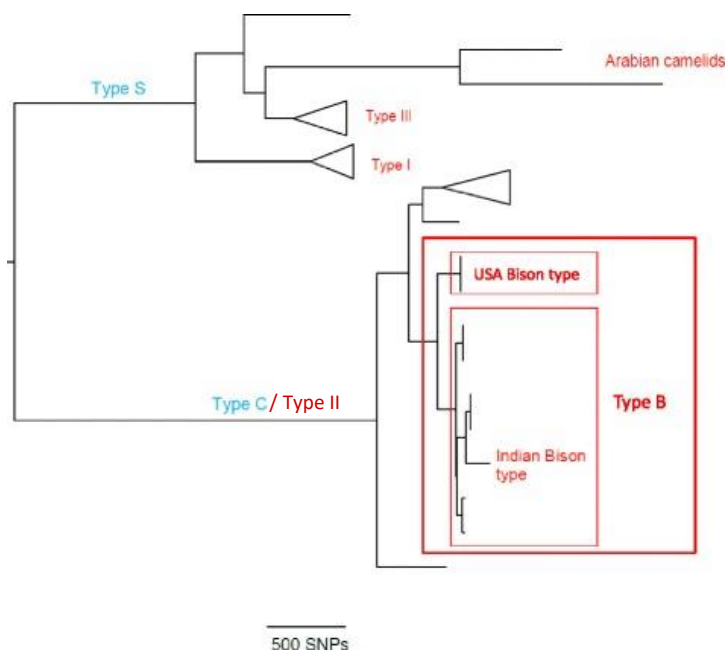


Figure 2: MAP strain type phylogeny (Stevenson, 2015; copyright Karen Stevenson. Reproduced under Creative Commons licence from Veterinary Research).

Prolonged environmental MAP viability may contribute significantly to maintenance of herd infection (Figure 3) (Whittington *et al.*, 2004). Viable MAP was demonstrated in faeces exposed to rain and sun 21 weeks after infection, with survival to 29 weeks in a moist environment with intermittent freezing (Lovell, Levi and Francis, 1944). Survival in slurry has been reported as reduced with increasing temperature (Olsen, Jørgensen and Nansen, 1985) and when mixed with urine (Larsen, Merkal and Vardaman, 1956). Type 1 and 3 MAP are reported to survive in shaded pasture for up to 55 weeks, with 32 week survival in unshaded

plots and 2 week survival in areas without vegetation or shade (Whittington *et al.*, 2004). Elongated survival in water or shaded sediment has been reported for up to 48 weeks or 36 weeks in a semi-exposed location (Whittington, Marsh and Reddacliff, 2005).

Ensiling of conserved forage at high pH has been demonstrated to result in MAP survival from 5 to 37 degrees Celsius for up to 14 days (Khol *et al.*, 2010; Elliott *et al.*, 2015). MAP survival in farm biogas plants has been described as extending for 2 to 6 months (Slana *et al.*, 2011), being positively associated with outdoor manure storage (Johnson-Ifeorundu and Kaneene, 1999).

Acidic soil increases iron availability which is hypothesised to increase MAP iron use or soil binding (Elliott *et al.*, 2015). For every 0.1 pH unit increase in soil pH, a 5% decrease in herd MAP prevalence was reported, with lime application to pasture resulting in a 72% decrease in MAP prevalence (Johnson-Ifeorundu and Kaneene, 1999).

MAP persistence in amitraz cattle dip with a pH of 12.4 for more than 14 days has been documented (Eamens, Spence and Turner, 2001), with survival at -70 °C in faeces for 15 weeks (Richards, 1981) and at +80 °C for 90 days demonstrated (Tkachuk *et al.*, 2013). Viable MAP has also been isolated in environmental dust on commercial farms (Eisenberg *et al.*, 2011).

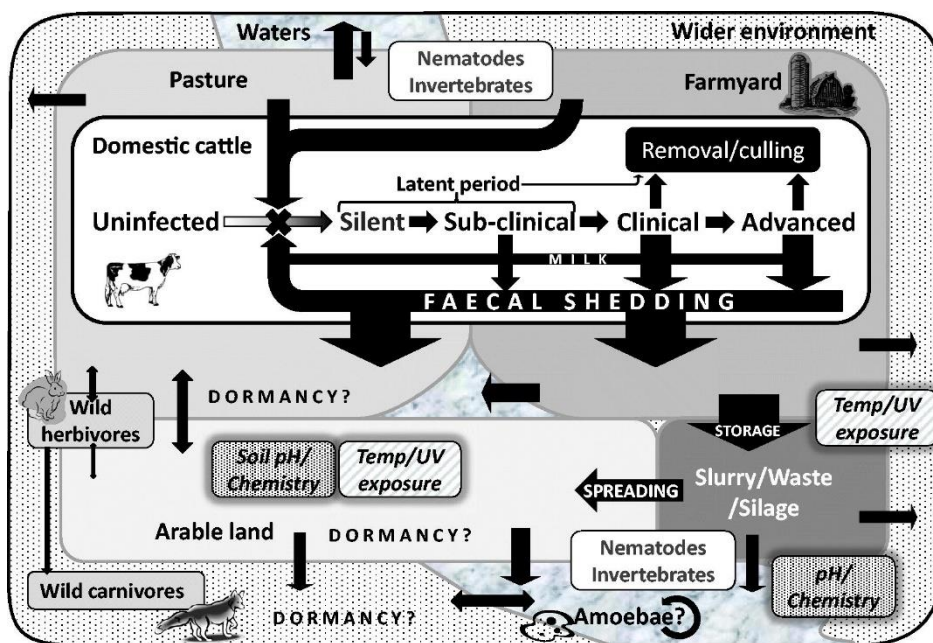


Figure 3: Environmental distribution of MAP (Elliott *et al.*, 2015; permission to reuse granted from Taylor & Francis).

1.3 Paratuberculosis transmission, pathogenesis and clinical signs

a. Routes of infection and infectious dose

Faeco-oral route during the neonatal period is the most common infection route with MAP bacteria in cattle (Arsenault *et al.*, 2014). The neonatal calf becomes contaminated with faecal origin MAP during the calving and post-partum period, with up to 80% transmission

from colostrum most likely from faecal contamination, including that associated with poor udder hygiene practices, rather than from MAP bacteria being directly shed into colostrum during colostrogenesis (Pithua *et al.*, 2011; Beaver *et al.*, 2017). Calves born from MAP negative dams are reported to be at an equally high risk of infection when housed in a MAP contaminated environment (Eisenberg, Rutten and Koets, 2015), with exposure to an increased MAP dose hastening development of clinical signs and gross intestinal lesions (Mortier, Barkema and De Buck, 2015). Minimum dose for experimental infection has been suggested as 10^9 CFU, administered via two consecutive daily doses, however significant variation exists on dose, strain and route of administration in literature, with consensus less clear on dose for successful natural infection (Hines *et al.*, 2007).

MAP bacteria are also shed in milk and colostrum from MAP infected cows (Stabel, 2008), with highest levels shed in clinically affected animals (250 CFU/ml) versus sub-clinically affected animals (24 CFU/ml), with most sub-clinical cows not shedding via this route at all (Khol *et al.*, 2013). Shedding was documented as highest in the first 60 DIM, reducing to negligible levels in the mid to late lactation (Stabel *et al.*, 2014).

Up to 9% of calves born from sub-clinically affected and 39% of calves born from clinically affected cows are reported to become infected before birth (Whittington and Windsor, 2009). Calves born from MAP seropositive dams at the time of calving were 6.6 times more likely to be MAP seropositive than calves born to MAP seronegative dams at the time of calving (Aly and Thurmond, 2005), although such calves may be exposed to higher quantities of MAP via dam colostrum and faeces during the peri-parturient period (Donat *et al.*, 2016), rather than in utero transmission. More recently, calves were reported to be 3.6 and 2.8 times more likely to test MAP positive if the dam seroconverted within 12 months or after 12 months post-partum, respectively (Patterson *et al.*, 2019).

MAP transmission has also been identified from calf-to-calf, with an R_0 of MAP transmission estimated as 0.1 to 3.2 between calves (van Roermund *et al.*, 2007) and 0.9 to 3.24 between group housed dairy calves, dependent on diagnostic criteria and whether final size or generalised linear modelling was used, in more recent work (Corbett *et al.*, 2019). Calves that were experimentally infected orally with MAP have been demonstrated to successfully infect calves housed in contact for 3 months, resulting in faecal shed MAP in contact exposed calves and MAP positive tissue samples in 50% of calves in this group (Corbett *et al.*, 2017).

Successful MAP transmission from experimental respiratory tract challenge with an inoculum of aerosolised MAP has been confirmed (Eisenberg *et al.*, 2011), although this route of transmission has not been demonstrated in the field. However, viable MAP bacteria has been identified in settled dust samples collected from Dutch dairy farms (Eisenberg *et al.*, 2010), with increased prevalence of MAP-positive dust samples associated with increased within-herd MAP infection prevalence (Eisenberg *et al.*, 2013a). Furthermore, MAP has been detected in the semen of clinically and subclinically affected bulls (Ayele *et al.*, 2004). MAP survives antimicrobial and cryopreservation techniques, demonstrating viability in AI straws (Givens, 2018), with its presence demonstrated to decrease sperm motility and vigour, reducing risk of successful fertilisation (Caldeira *et al.*, 2021). MAP

transmission has also been reported via saliva of infected adult cattle (Sorge, Kurnick and Sreevatsan, 2013) and during the embryo transfer process (Rohde and Shulaw, 1990), although no evidence of MAP bacteria was found in oocytes or embryos collected from low to moderate MAP shedding cattle in vivo following oocyte aspiration and embryo flushing, respectively (Kruip *et al.*, 2003).

Cross-species transmission has been reported, with evidence of MAP transmission from sheep to cattle on farms with high prevalence of ovine paratuberculosis or in New Zealand pastoral systems (Verdugo *et al.*, 2014). MAP bacteria have been detected in multiple non-farmed species (Bryant *et al.*, 2016), infecting and impacting 178 vertebrate species in total, however only five species are classified as true MAP reservoirs, including four species of deer and also rabbits (Carta *et al.*, 2013). European wild rabbits specifically experience a high prevalence of infection and can excrete up to 7.6×10^5 CFU/gram in faeces, although this is lower than in clinically affected cattle (Daniels *et al.*, 2003), reducing relevance unless there is potential for introduction of the infection into a naïve herd (Corn *et al.*, 2005).

Establishing the relative influence of each different transmission route is critical to effective control of infection on farm (McAloon *et al.*, 2019a). Infection from colostrum has been reported as possible but difficult to separate from other main infection routes (Nunney *et al.*, 2023). Currently, calves from infected dams regardless of test status at parturition and poor calving yard hygiene have particular proven positive associations with MAP infection risk (Patterson *et al.*, 2019; Nunney *et al.*, 2023), with a proportion of MAP positive animals unexposed to any measured risk factors (Nunney *et al.*, 2023), suggesting presence of unidentified factors that may contribute to MAP infection.

b. Susceptibility to infection

Age susceptibility of calves and young adult cattle to MAP infection decreases with increasing age; 75% calves less than 6 months old developed lesions consistent with paratuberculosis following a proven MAP exposure route, with 50% youngstock aged 6 to 12 months and less than 20% of adults aged over 12 months developing lesions following exposure (Windsor and Whittington, 2010). Increased intestinal permeability to allow colostral immunoglobulin and other macromolecule transfer early in life has been considered as causative in calves (Sweeney, 1996), as has increased circulating lymphocytes in neonatal calves promoting cellular immune response and suppressing macrophage activation (Chiodini, 1996).

Despite a functional rumen being hypothesised as protective (Windsor and Whittington, 2010), adult cattle can become clinically infected with MAP, being more common in highly contaminated environments (Rankin, 1961) or alongside nutritional or other stresses (Windsor and Whittington, 2010). This is particularly relevant for heifers grazing pasture contaminated with MAP from slurry (Mortier *et al.*, 2013).

Channel island breeds of cattle are reported to have increased susceptibility to MAP infection (Sorge *et al.*, 2011a), however other interacting herd factors such as nutritional management or location may have confounded these results. Genome wide association studies linking MAP infection status and different genetic loci have revealed associations of

eleven chromosomal QTL with the MAP infected phenotype (Minozzi *et al.*, 2012), although more recent studies found only three QTL significantly associated with MAP tissue infection (Kiser *et al.*, 2017).

c. Onset of infection (Figure 4)

MAP tissue invasion

Increased efficiency of calf intestinal epithelial cell infection is demonstrated to occur if MAP is pre-incubated in mammary epithelial cells or milk in the shedding animal (Patel *et al.*, 2006), due to increased activation of virulence factors and other specific genes of unclear function (Bannantine *et al.*, 2004). The oral mucosa in tonsillar crypts has been shown to be a route for MAP infection but mostly at higher doses (Payne and Rankin, 1961), with subsequent haematological spread to the mesenteric lymph nodes and ileum, considered as the primary site of MAP infection (Sweeney *et al.*, 2006). The location of MAP entrance may determine if MAP persistence or clearance prevails (Arsenault *et al.*, 2014).

MAP intestinal infection

Significant mucosal immune response has been observed in specifically the jejunum and ileum in neonatal calves (Charavaryamath *et al.*, 2013). MAP invasion occurs via the ileum within 30 minutes of contact, through M-cells of the Peyer's patch, alongside differentiated epithelial cells (Momotani *et al.*, 1988). This occurs following opsonisation of the bacteria by fibronectin which is activated by MAP gastrointestinal transit, attaching MAP to intestinal M-cells via fibronectin B1 receptors (Bannantine and Bermudez, 2013).

Macrophage invasion by MAP and establishment of 'controlled' infection

Complement, immunoglobulin, mannose and scavenger receptors mediate the sub-epithelial phagocytosis of MAP (Guirado, Schlesinger and Kaplan, 2013), with complement receptors shown to reduce macrophage activation and increase MAP survival (Premanandan *et al.*, 2009). Following ileal infection, it is documented to take 5 hours for phagocytosis of MAP bacteria (Momotani *et al.*, 1988), with macrophage killing of ingested MAP organisms critical at this stage of invasion to reducing risk of chronic infection establishment. Th1-type T-helper lymphocytes produce cytokines such as interferon-gamma, to attempt intracellular killing of MAP (Mortier, Barkema and De Buck, 2015). If MAP is not eliminated via this mechanism, an extended phase of controlled infection follows, which may last for 2 or more years following initial macrophage invasion. Toll-like receptors (TLRs) expressed on sentinel cells recognise invading MAP and initiate immune response and cytokine release, however, individual mutations in this system can result in increased predisposition to successful MAP infestation (Mucha *et al.*, 2009). TLR-9 signalling has been shown to be redirected by MAP to reduce intracellular bacterial clearance and increase chronicity of infection (Arsenault *et al.*, 2012).

MAP induced subversion of macrophages enables progressive development of a persistent infection, inhibiting phagosome acidification and fusion, preventing bacterial death via hydrolysis and oxidation (Koul *et al.*, 2004).

Localised granulomas progressively develop, made up of giant cells, macrophages, lymphocytes and epithelioid cells which contain MAP in focal sites (González *et al.*, 2005).

There are no detectable clinical signs of infection, subclinical impacts, faecal MAP shedding or raised serum antibody titre, with measures of cell mediated cytokines such as interferon-gamma the only potentially demonstrable substance. Sampling the ileum or mesenteric lymph node for bacteriology or PCR is the only dependable method for MAP detection at this stage of disease (Sweeney, 2011).

d. Progression of infection

Subclinical stage

Transition from cell-mediated immunity to an ineffective humoral, Th2-type immune response occurs after a variable period of time, associated with an increase in IL-4 and IL-10 production (Stabel, 2000a). This transition is commonly referred to as a loss of 'control' of infection, however, the instigating cause is unclear (Mortier, Barkema and De Buck, 2015) and may be secondary to an accumulation of extracellular bacteria as a result of granulomatous disruption to the intestinal mucosal structure (Magombedze, Eda and Ganusov, 2014). Faecal MAP shedding increases, with progressive spread of MAP bacteria to other organs such as the uterus, mammary gland and muscle (Seitz *et al.*, 1989; Sweeney, Whitlock and Rosenberger, 1992; Streeter *et al.*, 1995). Antibodies generally become detectable by ELISA following the advent of faecal MAP shedding (Sweeney *et al.*, 2006). Although there are no signs of clinical disease, significant subclinical impact on performance and production at cow and herd level has been documented at this point, reviewed in more detail in section 1.7.

Clinical stage

Development of clinical signs is typically described after two years of age (Nielsen and Toft, 2006). Speed of progression to the clinical stage of disease from the onset of faecal MAP shedding is variable and may occur after stressful events such as parturition, nutritional or metabolic challenges or infection with other agents *i.e. fasciola hepatica* (Naranjo Lucena *et al.*, 2017). Granulomatous intestinal lesions become diffuse and spread to impact the jejunum, ileum, caecum and colon (Buergelt *et al.*, 1978), with thickening of the intestinal villi with granulomatous infiltration and the development of granulomatous lymphadenitis with multiple intracellular MAP bacteria visible on histopathology, significantly reducing intestinal absorptive capacity (González *et al.*, 2005). Clinically, cattle suffer a malabsorptive 'pipe-stream' diarrhoea and subsequent protein losing enteropathy, weight loss and subcutaneous oedema, prominent in the brisket or submandibular regions. Milk yield is significantly reduced, with common dissemination of MAP to extraintestinal sites at this stage. If progression of disease is allowed, fatal cachexia and dehydration ensues (Fecteau, 2018).

The 'iceberg phenomenon' is widely used to describe the epidemiological pattern of paratuberculosis in the herd (Whitlock and Buergelt, 1996), although proportions of pre/sub-clinically affected animals relative to clinical cases may be lower than previously suggested (Magombedze, Ngonghala and Lanzas, 2013).

Additional forms of paratuberculosis have been proposed; animals may become infected and appear resistant with no histopathological lesions or bacteriological evidence of MAP at necropsy or may recover completely with sterile granuloma lesions but no bacteriological

evidence of MAP bacteria (Whittington *et al.*, 2017). This has been rarely demonstrated in literature (Dennis, Reddacliff and Whittington, 2011).

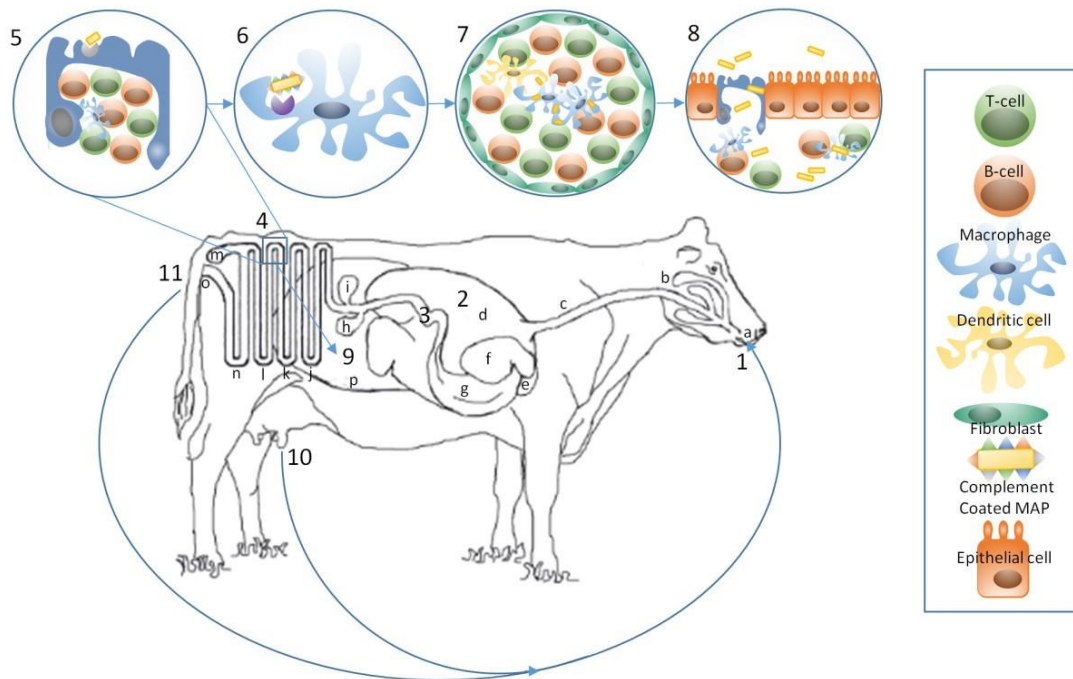


Figure 4: MAP ingestion and infection routes; 1- oral ingestion, 2-ruminal activation and opsonisation, 3- intestinal transit, 4/5- ileal M cell uptake in Peyer's patches, 6- macrophage phagocytosis of MAP, 7- granuloma formation by infected macrophages, 8- active paratuberculosis, 9/10- priming of mammary glands and milk with MAP during active MAP shedding, a- mouth, b- salivary glands, c- oesophagus, d- rumen, e- reticulum, f- omasum, g- abomasum, h- gallbladder, i- pancreas, j- duodenum, k- jejunum, l- ileum, m- caecum, n- large intestine, o- anus, p- uterus (Arsenault *et al.*, 2014; copyright Ryan Arsenault. Reproduced under Creative Commons licence from Veterinary Research).

e. MAP shedding patterns

Most faecally shedding cattle shed low amounts of faecal MAP, with 71% cattle shedding less than 5 CFU per gram, 10% shedding 5 to 25 CFU per gram and only 19% classified as high shedders, shedding more than 25 CFU per gram of faeces (Whitlock *et al.*, 2000). In one study, over 95% of cattle shedding over 100 CFU per gram of faeces had been culled or died within 12 months of faecal sampling (Mitchell *et al.*, 2015). Infected cattle either remain shedding low levels of MAP, intermittently with an absent or low level of humoral response, being classified as 'non-progressors' or progress to continually shedding increasing levels of MAP bacteria, categorised as 'progressors' (Mitchell *et al.*, 2015).

'Super-shedders' have been described; these animals shed more than 10^7 CFU MAP per gram of faeces, capable of infecting more than 1,000 calves per gram (Aly *et al.*, 2012). Prevalence of super-shedders was only estimated as 0.5% in one large herd, however

represented 10% of PCR positive and 14% of serum ELISA positive cows (Aly *et al.*, 2012). The consensus on the risk of MAP spread in a herd from super-shedders is unclear; some work has demonstrated that a 1000 times increase in MAP load shed only causes a 2 to 3 times increase in level of MAP spread in a herd (Slater *et al.*, 2016), however other work has reported that presence of MAP infected cattle with the super-shedding phenotype strongly influenced MAP spread in a herd (Ben Romdhane *et al.*, 2017). Temporal clustering of ELISA positive cases has been shown to be present in a number of US herds (Zare *et al.*, 2013), potentially indicating the role of a super-shedder in generation of these clusters.

1.4 Zoonotic impact

a. Crohn's Disease

Crohn's Disease is a chronic, inflammatory gastroenteritis requiring lifelong management (Naser *et al.*, 2014), with unknown aetiology. Genetic susceptibility is suspected to induce a dysregulated immune response towards commensal microbial populations, alongside environmental factors.

MAP DNA was isolated from intestinal mucosa and blood of Crohn's Disease patients (Mishina *et al.*, 1996; Naser *et al.*, 2004). An extensive systematic review meta-analysis reported that Crohn's patients were between 4.3 and 8.4 times more likely to have MAP isolated compared to ulcerative colitis, inflammatory bowel disease and healthy patient controls, regardless of diagnostic method (Waddell *et al.*, 2015). MAP has also been isolated in intestinal histopathology samples from patients with ulcerative colitis and colorectal cancer as well (Pierce, 2018).

MAP isolates detected in both humans and cattle have demonstrated very close phylogenetic relationships in the USA and the Netherlands (Bryant *et al.*, 2016), with strains being reported as identical in India (Chaubey *et al.*, 2017). Despite the presence of MAP in affected patients, shared MAP strain similarity and clear routes of exposure between cattle and humans, there is insufficient evidence for successful MAP transmission from infected cattle and of the exact role of MAP in Crohn's Disease (Waddell *et al.*, 2016), making public health implications difficult to characterise.

b. Other diseases

Patients with type 1 diabetes are reported 2.9 fold more likely to have MAP detected by PCR and 9.9 fold more likely to respond with an immune reaction to MAP as detected by ELISA (Waddell *et al.*, 2015). Very weak evidence is present for an association between multiple sclerosis and MAP infection (Frau *et al.*, 2013), with this association only reported in this population with high levels of multiple sclerosis, hence reducing generalisability. Further research is still required for the developing hypothesis that there may be an association between Hashimoto's thyroiditis and MAP infection (Masala *et al.*, 2014).

Significant effects of herd infection have been documented on farmer mental health and wellbeing, with particular impact of clinical cases (McAloon *et al.*, 2017a).

c. Human access to MAP sources

Presence of viable MAP has been confirmed in locally and commercially pasteurised and raw milk in the UK (Grant *et al.*, 2002; Grant, Ball and Rowe, 2002) and worldwide (Ayele *et al.*, 2005). Detection of viable MAP has been established also in powdered infant formula milk confirmed by culture (Botsaris *et al.*, 2016) and in UK post-treatment water samples diagnosed by PCR and culture (Aboagye and Rowe, 2011). Viable MAP has also been reported present in raw and pasteurised milk cheese (Waddell *et al.*, 2016), in diaphragm muscle of infected cattle (Alonso-Hearn *et al.*, 2009) and present in 80% of beef burgers containing lymphatic tissue from a MAP infected cow (Mutharia *et al.*, 2010). MAP was still detected by culture and PCR from contaminated ground beef after freezing to both -18°C and -196°C (Mutharia *et al.*, 2010), however a 12 log cycle reduction in MAP bacteria was reported after cooking contaminated ground beef for 1.5 minutes at 71.1 °C or above (Saucier and Plamondon, 2011).

Prevalence of foodborne MAP represents a significant potential public health concern, and viability of the bacteria after pasteurisation, processing and food preparation demonstrates the value of reducing MAP prevalence at farm level and the use of a control plan centred on HACCP principles to help manage risk on farm (McAloon *et al.*, 2015). Furthermore, this could risk the reputation of the global dairy industry if consumer perception is mismanaged (Groenendaal and Zagmutt, 2008) or on farm control is not prioritised (Kuenstner *et al.*, 2017).

1.5 Diagnostic testing

Accurate diagnosis of MAP infection proves challenging, with test performance dependent on disease stage and variable, low sensitivity (ability to correctly identify animals in the target disease stage as positive) and specificity (ability to correctly identify animals not in the target disease stage as negative) of currently available tests at animal level (Nielsen and Toft, 2008). Animals are recognised as infected with no clinical signs or faecal shedding, as infectious with faecal shedding but no clinical signs/subclinical impacts only or as affected with clinical signs as well as faecal shedding (Nielsen and Toft, 2008). Whole herd testing can prove more relevant in classifying herd infection status and estimating prevalence (Field, Mee and McAloon, 2022).

a. Individual animal

Interferon gamma

Presence of this substance can indicate subclinical MAP infection or recent exposure to MAP, as it is released by sensitised lymphocytes during a cellular immune response after in vitro stimulation with mycobacterial antigens, such as Johnin PPD and *M. bovis* PPD (Stabel, 2000b). This assay is best suited to diagnosing recent MAP infections, however test characteristics have been demonstrated to be dependent on PPD type, diagnostic criteria used and age of animal (Jungersen *et al.*, 2002).

In calves, a peak in response was demonstrated 4 months after experimental inoculation with MAP, with animals exposed to a higher MAP dose producing an earlier and higher

interferon gamma response compared to those exposed to a lower MAP dose (Mortier *et al.*, 2014a).

This assay is rarely used in the field; test characteristics were initially determined on infectious not infected cattle and a small number of herds reducing generalisability of results, with a lack of commercial availability (Nielsen and Toft, 2008).

MAP antibody detection

An absorbed indirect ELISA is used for this test, measuring IgG1 antibody titre against MAP antigen, however antigen preparations used in different tests are often not comparable (Nielsen and Toft, 2008). Detectable antibody titre is recorded from a minimum of 10 months after infection (Lepper *et al.*, 1989), with age at sero-conversion recorded from 2 to 11 years (Nielsen and Ersbøll, 2006) and the probability of detecting infected cattle by antibody ELISA at 2 years of age estimated at 0.06 (Nielsen and Toft, 2006). However, antibody titres have been documented to increase and decrease over time in calves in the first six months after infection, with the combined use of serum ELISA and faecal culture suggested for use in youngstock in testing programmes, alongside the routine ELISA screening of cattle more than 24 months of age (Mortier, Barkema and De Buck, 2015).

Due to the chronic nature of the disease, the sensitivity of the milk and serum ELISA increases as disease develops (Nielsen and Toft, 2008), with sensitivity at subclinical stages of disease estimated to be 15%, rising to more than 90% in clinical disease stages (Collins *et al.*, 2005). Increasing antibody ELISA test frequency maximises the ability to detect MAP infection as disease progresses (Nielsen and Toft, 2006). Furthermore, assessing individual antibody ELISA results in the context of subclinical production impact, clinical signs and herd MAP infection prevalence is vital (Nielsen and Toft, 2009), with an increased positive predictive value for a positive ELISA result more likely in clinical or high prevalence cases (Bender *et al.*, 1998).

- **Blood antibody ELISA**

This serum test is commonly used commercially, particularly in beef herds, as cheap and practical to use (Collins, 2011). Test characteristics documented are dependent on disease stage; an infected animal is defined as infected with MAP bacteria and presenting a risk of becoming infectious in future, an infectious animal is defined as currently shedding MAP bacteria presenting risk of transmission to susceptible animals and an affected animal is defined as demonstrating clinical signs of paratuberculosis, in addition to being variably infectious (Nielsen and Toft, 2008). For detection of infected cattle, sensitivity is reported from 7% to 22% and specificity as 91 to 98%, for detection of infectious cattle sensitivity is reported from 24% to 94% and specificity from 41% to 100%, for detection of affected cattle sensitivity is reported from 50% to 87%, with specificity not reported (Nielsen and Toft, 2008). Interpretation of serum antibody ELISA results has been performed with application of likelihood ratios to predict odds of MAP infection and stage of infection. Cattle with a serum antibody ELISA s/p ratio of 0.10 to 0.24 have evidence of an antibody titre above normal baseline levels and are 5 to 15 times more likely to be MAP infected. Cattle with a serum antibody ELISA s/p ratio of 0.25 to 0.39 are classified as weakly positive, with a 16-fold increase in likelihood of being MAP infected. Cattle with a serum antibody s/p ratio of

0.4 to 0.99 are classified as having a moderate antibody titre, being 30 times more likely to be MAP infected and be shedding MAP in faeces and milk. Finally, cattle with a serum antibody s/p ratio of 1 or more are over 200 times more likely to be MAP infected and in advanced disease stages, with significant faecal and milk shedding of MAP (Collins, 2002).

- **Milk antibody ELISA**

This is a quick and cost-effective method for sampling dairy herds, by either the farmer during the milking or by a milk recorder on a routine basis, using milk taken for milk quality testing. Compared to blood antibody ELISA, sensitivity estimates for detection of infectious and infected animals are lower (29% to 61%), specificity estimates for these infection categories are higher (83% to 100%), with the test not evaluated for clinically affected animals (Nielsen and Toft, 2008). Milk antibody ELISA results can be used to predict subsequent risk of faecal shedding and infectiousness; 70% of cattle classified as high faecal shedders tested positive by milk antibody ELISA at the commencement of faecal MAP shedding, with 28% testing positive a year or more prior to this. A lesser proportion of cattle with low (60%), transient (5%) or intermittent (30%) faecal shedding patterns were also milk antibody ELISA positive at the commencement of faecal shedding (Nielsen, 2008).

Increased yield has been demonstrated to decrease the detected milk antibody titre, with milk testing suggested to be performed in either early or late lactation when milk yield is lowest (Eisenberg *et al.*, 2015).

- **Impact of tuberculin testing**

Initial work established that following performance of the SICCT for bovine tuberculosis, MAP antibody ELISA values remained significantly higher until day 43 post SICCT in milk and day 71 in serum (Kennedy *et al.*, 2014). More recent work similarly concluded that performing a MAP antibody ELISA test within 60 days of a SICCT increased risk of a positive MAP antibody ELISA result, however also found that the proportion of cattle testing subsequently negative after the first positive MAP antibody ELISA test was greater if the first positive test was within 30 days of the SICCT rather than more than 90 days after (Barden, Smith and Higgins, 2020). This suggests that such cattle were 'falsely' positive and reflects a small reduction in antibody ELISA specificity. More recently it was suggested that this effect differs between MAP infected and non-MAP infected cows; MAP infected cows had the highest milk antibody ELISA value closest to SICCT testing, suggesting an anamnestic effect following the injection of avian PPD as part of SICCT testing in infected cattle, increasing MAP antibody ELISA sensitivity when performed subsequent to SICCT testing (Nunney *et al.*, 2022). Non-MAP infected cows also showed an increase in MAP antibody ELISA titre following SICCT testing resulting in a small reduction in test specificity when MAP testing was performed between 15 to 28 days post SICCT testing, hypothesised to be a primary immune response following avian and bovine PPD injected during the SICCT (Nunney *et al.*, 2022).

Culture

Bacteriological culture of MAP is the definitive diagnostic method for confirming paratuberculosis infection and verifies presence of viable bacteria with infectious potential and strain differentiation (Whittington *et al.*, 2017). An incubation period of 12 to 20 weeks

on solid media and 8 to 12 weeks on liquid media is required (Schwalm *et al.*, 2018), followed by phenotypic and genotypic identification (Ricchi *et al.*, 2014). MAP can also be bacteriologically detected in samples of intestine, lymph node (Chiodini, 1989) or blood (Bower, Begg and Whittington, 2011), red and processed meat (Mutharia *et al.*, 2010) and milk and dairy products (Donaghy, Totton and Rowe, 2003; Gao *et al.*, 2005), as well as faeces.

Faecal culture is widely deemed to be the gold standard for confirmatory diagnosis of paratuberculosis (Whitlock *et al.*, 2000), with specificity of almost 100% if isolates from culture are confirmed as containing the IS900 insertion sequence on PCR (Nielsen and Toft, 2008). However, cattle may ingest feed or water contaminated with MAP infected faeces and begin faecally shedding MAP in high prevalence herds (Sweeney *et al.*, 1992), leading to false-positive faecal culture results, and reducing specificity to 98% (Nielsen *et al.*, 2002).

The sensitivity of faecal culture is reported from 33% to 70% overall (Whitlock *et al.*, 2000). Many studies did not follow animals for a prolonged period of time and many had younger cohorts, increasing the chance of sampling intermittent shedders in periods of non-MAP shedding, resulting in false-negative faecal culture results (Nielsen and Toft, 2008). For detection of infected animals sensitivity has been estimated at 23% to 29%, with sensitivity for detection of infectious and affected animals reported as 74% and 70%, respectively (Nielsen and Toft, 2008).

Early life faecal culture may have value in improving efficacy of control programs; faecal MAP shedding has been documented in 61% of calves two weeks after MAP infection, being the first test to become positive (Mortier *et al.*, 2014b; Mortier, Barkema and De Buck, 2015), peaking one to six months later (Subharat *et al.*, 2012). It is suspected that results may become positive again after a test-negative period (Stabel, 2006).

PCR

PCR diagnoses MAP infection by detection and amplification of nucleic acid within MAP DNA in a sample, such as faeces, milk, tissue or meat, semen, blood and lymph fluid (Collins, 2011). This method has a faster turnaround time than culture and enables quantification of MAP faecal shedding levels with good correlation to faecal culture results (Aly *et al.*, 2010). Quantification of MAP DNA and therefore levels of bacteria is achieved via assessment of the Ct value, using real-time quantitative PCR. Heavy shedding has been classified by a quantitative PCR Ct value of less than 29, with moderate or light shedding defined by a quantitative PCR Ct value of less than 35 (Laurin *et al.*, 2015), although these thresholds may fluctuate between differing quantitative PCR kits.

Strain differentiation of organisms is not possible with PCR testing alone without sequencing. Similar to bacteriology, interpretation of low levels of MAP DNA quantities in high prevalence herds may be as a result of the 'pass-through' phenomenon of MAP bacteria consumed in feed and water by cattle, rather than active shedding (Sweeney *et al.*, 1992), especially considering the capacity of PCR to detect DNA from non-viable bacteria (Navarro-Gonzalez *et al.*, 2019). However, considering the reduced sensitivity of faecal culture in low shedding animals, passive shedders may represent truly infected low

shedding cattle 'missed' by this reference test (Navarro-Gonzalez *et al.*, 2019), especially as passive and low shedding cattle are reported to shed comparable amounts of MAP (Kralik *et al.*, 2014).

Test characteristics vary significantly, differences in performance may be due to variation in sample types used (*i.e.* naturally infected or experimentally spiked faecal samples), in extraction and purification methods used, in the PCR test kits used and DNA fragment targeted (Meyer *et al.*, 2019). Sensitivity at animal level is still hampered by the intermittent faecal MAP shedding, difficulty extracting MAP DNA through the complex cell wall (Timms, Mitchell and Neilan, 2015) and contaminating faecal substances inhibiting DNA amplification (Schrader *et al.*, 2012). Specificity is comparable to bacteriology although reduced in high prevalence herds (Kralik *et al.*, 2014), with sensitivity varying from 50% to 70% (Plain *et al.*, 2014; Acharya *et al.*, 2017). The main DNA target sequences used are IS900 and ISMAP02 (Green *et al.*, 1989; Stabel and Bannantine, 2005), other MAP specific genes are used to increase specificity of PCR assays however have less copy numbers associated with them in the MAP genome (Bannantine *et al.*, 2002).

Gross pathology

Lesions identified on gross pathological examination post mortem are most likely recognised late in the disease process, with a sensitivity of 40 to 70% and specificity of 80 to 95% estimated for true disease detection alone (Whittington *et al.*, 2017). Examining a greater number of intestinal sites examined increases sensitivity of disease detection (Whitlock, Rosenberger, and Sweeney, 1996). Gross lesions notable on necropsy include focal or diffuse intestinal inflammation, with obvious mural oedema and mucosal thickening with transverse folds (Buergelt *et al.*, 1978). Draining mesenteric lymph nodes are pale, enlarged and oedematous with dilated connecting lymphatic vessels in serosa (Whitlock and Buergelt, 1996). Macroscopic pathology is most commonly isolated to the ileum, however the liver and hepatic lymph nodes may develop similar lesions, alongside degeneration of mesenteric fat, anaemia and alopecia in cachectic animals, with aortic mineralisation (Whitlock and Buergelt, 1996). Lesion severity may not always reflect the stage of clinical disease and gross pathology is not specific to paratuberculosis alone (Buergelt *et al.*, 1978).

Histopathology

Initial sampling of the terminal ileum, tissue surrounding the ileo-caecal valve and local lymph nodes is advised, with more diffuse intestinal samples possible in advanced disease (González *et al.*, 2005).

Detection of subtle earlier disease stages is possible alongside confirmation of gross pathological change, with specificity reported as 95% (Martinson *et al.*, 2008). Varying sensitivity estimates from 0 to 100% are described, however significant variation existed between studies in sampling rationale, patient age and stage of disease sampled (Martinson *et al.*, 2008).

A range of classification systems describing lesion extent, severity and type are present, enabling definition of disease stage (Whittington *et al.*, 2017). The Ziehl-Neelsen stain has been used histologically to identify the bacilli cell wall of MAP, however will not stain

bacteria without an intact cell wall and cannot differentiate different mycobacterial species or other bacteria such as *Cornynebacterium* which also stain acid-fast (Brees *et al.*, 2000). A range of immunohistochemical techniques can also be used, with equivalent or increased sensitivity and specificity compared to Ziehl-Neelsen staining (Brees *et al.*, 2000).

Developing diagnostic methods

Use of novel MAP specific antigens that provoke an earlier immune response has improved success in detecting MAP infected cattle by ELISA when compared to using a commercial ELISA (Mon *et al.*, 2012) and improved the specificity of the interferon-gamma assay (Mikkelsen *et al.*, 2011).

The FASTPlaque TB bacteriophage assay has been demonstrated to identify 1 to 10 CFU/ml of MAP in milk within 48 hours of incubation, detecting clinical and subclinically affected animals (Foddai, Elliott and Grant, 2009; Swift *et al.*, 2013, 2016). Sensitivity and specificity have been estimated as 35% and 100% respectively in milk, when compared to faecal bacteriology or milk antibody ELISA results (O'Brien *et al.*, 2018).

Differences in the faecal microbiome have also been documented in MAP infected cattle compared to uninfected cattle (Derakhshani *et al.*, 2014), although diet and sampling location can impact the contents of the gastrointestinal microbiome in cattle (De Menezes *et al.*, 2011; Fouts *et al.*, 2012). Furthermore, in MAP infected cattle, plasma protein concentrations were significantly different for eight different proteins, with six proteins upregulated and two proteins down regulated (You *et al.*, 2012).

Increased expression of mRNA in peripheral blood mononuclear cells promoting a Th2 phenotype and cellular apoptosis, has also been reported in MAP infected cattle (Coussens *et al.*, 2005), with MiRNA expression also demonstrated to increasingly differ with progression of MAP infection, with two particular miRNAs isolated as potential biomarkers during the subclinical disease stages (Choi *et al.*, 2021).

b. Herd level

Whole herd ELISA testing

Milk sampling is commonly completed every 3 months in dairy herds, due to low cost and as part of national control programs (Orpin, Sibley and Bond, 2020). Across milk and serum whole herd ELISA testing, with at least one seropositive animal in the herd defining a herd as positive, herd level sensitivity and specificity estimates range from 83% to 10% and 0 to 65.5% in literature (Field, Mee and McAloon, 2022). Increasing the seroprevalence associated with positivity to $\geq 2\%$, improved herd level specificity to 96% for the milk ELISA (Lavers *et al.*, 2014). Follow up of seropositive animals with faecal culture or PCR has demonstrated varying performance depending on within-herd prevalence of MAP infection, often being performed to maximise herd level specificity (Field, Mee and McAloon, 2022).

Targeted or random serological sampling

Bi-annual targeted milk antibody ELISA screening of 30 high risk cows (low yield, high somatic cell count) is a minimum surveillance requirement in the UK National Johne's Management Plan (Orpin, Sibley and Bond, 2020). Using cow lactation number, somatic cell count and yield data to select 30 cows for milk antibody ELISA screening increased the

probability of detecting at least one positive cow in 95% of 385 dairy herds, compared to 84% if 60 cows were selected at random for sampling (Hanks, Taylor and Kossaibati, 2014).

Bulk milk tank ELISA testing

Using the manufacturer recommended s/p ratio, herd level sensitivity was reported as 8% to 30%, reducing value in herd-level diagnosis (Field, Mee and McAloon, 2022). This may be secondary to intermittent antibody shedding and reliance on a large enough number of diseased animals contributing to the pooled milk sample (Imada *et al.*, 2023), however, specificity estimates were high, at 95% to 100% (Field, Mee and McAloon, 2022), increasing trust for an initial positive herd result.

Whole herd individual faecal testing

Few studies have investigated the herd level sensitivity of individual faecal testing (bacteriology or PCR), however as sample size and whole herd prevalence increases, sensitivity is estimated to increase, up to 100% (Tavornpanich *et al.*, 2008).

Pooled faecal testing

Testing pools of five individual faecal samples is most commonly reported (Tavornpanich *et al.*, 2008), with herd level sensitivity estimates ranging from 26% to 100% and specificity ranging from 98% to 100% (Field, Mee and McAloon, 2022).

Bulk milk tank bacteriology, PCR and phage testing

Milk samples from the bulk milk tank or direct samples from the milk filter have been evaluated for detection of MAP bacteria, using bacteriology, PCR and phage testing in a number of studies (Stabel, Wells and Wagner, 2002; Slana *et al.*, 2012; Foddai and Grant, 2017). Estimates of herd level sensitivity for these tests ranges from 0 to 85%, with specificity ranging from 50 to 100% (Field, Mee and McAloon, 2022).

Environmental sampling

Samples from housing passageways, slurry tanks, parturition yards and sick cow pens, drain water and post-weaning calf accommodation were positive for MAP bacteria in 78% of 80 dairy herds sampled, with 80% of herds having at least one MAP positive environmental sample (Raizman *et al.*, 2004). The most frequently used procedure for environmental sampling is to collect six composite samples from cow housing, manure storage areas and other areas of high cow traffic (such as the collecting yard and sick cow pens) (Field, Mee and McAloon, 2022). Herd level sensitivity for this protocol ranges from 24% to 79%, with specificity estimated as 99% in one study (Field, Mee and McAloon, 2022). Boot swab sampling has also been examined in a range of studies; sensitivity estimates of 44% to 91% have been reported for mycobacterial culture of swabs (Eisenberg *et al.*, 2013b; Wolf *et al.*, 2016b; Hahn *et al.*, 2017), with PCR sensitivity and specificity estimates of 44% to 72% and 75% to 100%, respectively (Hahn *et al.*, 2017; Delafosse *et al.*, 2019). An environmental sampling protocol from areas of high cow traffic (such as alleyways, feed-face and calving pens) has been reported to successfully detect 9 out of 14 MAP positive beef suckler herds, demonstrating the value of this approach for initial herd screening (Klawonn *et al.*, 2016).

1.6 Prevalence of disease

Numerous cow-level and herd-level prevalence studies have been conducted worldwide with paratuberculosis considered endemic in areas of developed dairy industry (Garcia and Shalloo, 2015). Paratuberculosis is a listed disease notifiable to the WOAH (World Organisation for Animal Health, 2023) and is listed within category E to be kept under surveillance for farmed species under the EU Animal Health Law (European Union, 2016). In a recent review, it was reported that the disease is also notifiable in 73% of studied countries (35 out of 48) (Whittington *et al.*, 2019).

Prevalence of paratuberculosis is reported in two main forms in literature; apparent prevalence describes the ratio of the number of animals testing positive for disease to the number of animals tested, whilst true prevalence estimates adjust apparent prevalence estimates according to the accuracy and specificity of diagnostic testing used, varying under different conditions of use in some studies (Lombard, 2011).

a. Herd-level

Herd-level prevalence can be estimated from the proportion of herds in a sample with one or more MAP positive animal, at a given time. Differences in sampling and diagnostic strategy used can make prevalence estimates difficult to compare (Lombard, 2011).

World-wide

There are multiple countries where prevalence is unknown or inaccurate (Nielsen and Toft, 2009); under-reporting was suspected in 74% of countries studied in recent work, with lack of surveillance or national legislation, lack of knowledge and poor test characteristics, or reluctance to report results suggested as contributory (Whittington *et al.*, 2019). Despite this, paratuberculosis appears to be present world-wide, with at least 20% herds infected in 58% countries studied recently (Whittington *et al.*, 2019). Apparent herd-level prevalence is widely reported as >50% in North America and Europe, mostly for dairy herds (Nielsen and Toft, 2009; Lombard *et al.*, 2013). Nordic countries are examples of low apparent prevalence countries with use of notifiable disease controls, surveillance and vaccination, and high apparent prevalence countries include Denmark, Belgium, USA and the UK (Nielsen and Toft, 2009).

Herd-level prevalence of paratuberculosis in beef systems has been reported variably; only 7.9% of herds were deemed apparently infected in the USA in early work (Dargatz *et al.*, 2001), however much higher estimates of 34% to 76% and 15% to 28% have been documented more recently in the USA and Canada, respectively (Roussel *et al.*, 2005; Roussel, 2011).

Large and variable herd-level true prevalence estimates from 23%-91% are documented specifically from Bayesian modelling approaches (Pozzato *et al.*, 2011; Lombard *et al.*, 2013; Verdugo, Toft and Nielsen, 2015; McAloon *et al.*, 2016a), although it should be noted that over-estimation of true herd-level prevalence by such models has been reported, due to low diagnostic test sensitivity and within-herd true prevalence (McAloon *et al.*, 2019b).

United Kingdom

Dairy herd-level prevalence estimates for the UK are limited and varied; at least 75% herds have been reported with one apparently seropositive cow, with 2.1% herds estimated as truly free of disease in one study conducted in the south west of the UK (Woodbine *et al.*, 2009). Recent UK work estimated a 68% true prevalence of bulk milk antibodies against MAP in study herds (Velasova *et al.*, 2017), adjusting for the reduced herd-level sensitivity of this test. Smaller dairy herds (less than 100 cows) were associated with a reduced seroprevalence of infection, with seropositive cattle clustered by birth month in larger herds (more than 100 cows) (Woodbine *et al.*, 2009).

At least 42% suckler herds were reported with one apparently seropositive cow, with 21.1% suckler herds estimated to be truly free of infection during the study period (Woodbine *et al.*, 2009).

b. Cow-level

Word-wide

Animal level prevalence estimates reported are again higher for dairy than beef breeds and for values derived from testing cattle at slaughter or at market sales compared to on farm testing (Lombard, 2011). In Europe, apparent prevalence of MAP at largely dairy cow level ranges from 0% to 24%, however, many study designs used non-random sampling based upon clinical suspicion of disease or did not specify the sampling frame used, reducing certainty that generalisation of results to the entire cattle population of these countries can be applied (Nielsen and Toft, 2009). True prevalence of MAP infection in mostly dairy cattle in Europe has been estimated at around 20%, based upon studies in France, Germany, Italy and Turkey (Nielsen and Toft, 2009). Estimates from Latin America and the Caribbean demonstrated apparent animal level prevalence of 16.9% (Fernández-Silva, Correa-Valencia and Ramírez, 2014), with North American estimates ranging from 1% to 20% (Lombard, 2011).

For beef breeds, again few countries report animal level prevalence (Lombard, 2011); apparent cow level prevalence is estimated as ranging from 3% to 9.6% and 0.8% to 1.5% in the USA and Canada, respectively (Roussel *et al.*, 2005; Roussel, 2011), true prevalence was reported as 0.8% in beef cattle sampled at slaughter in Denmark (Okura, Nielsen and Toft, 2010) and 6.3% for beef cattle tested by serum antibody ELISA in Ireland (Good *et al.*, 2009).

United Kingdom

Literature is limited on animal level prevalence estimates for the United Kingdom, however, a slaughter-house based study in the South West of England reported a subclinical disease prevalence of 2.6% and 3.5% as identified by intestinal lymph node culture and PCR, respectively (Çetinkaya *et al.*, 1996).

1.7 Production and economic impacts

a. Milk yield

Reductions of mean milk yield from 500kg to 1,400 kg per cow have been estimated for the lactation when cows tested positive for MAP infection, by either faecal culture or serum

ELISA (Johnson *et al.*, 2001; Kudahl, Nielsen and Sørensen, 2004; Beaudeau *et al.*, 2007; Gonda *et al.*, 2007; Richardson and More, 2009). However, other work has found no change or an increase in milk yield in MAP positive cattle (Smith *et al.*, 2009; Kennedy *et al.*, 2016).

Milk yield reduction is reported as most prevalent in more advanced infection (Johnson *et al.*, 2001); faecal culture positive cows are documented to produce 1,355 kg less milk than faecal culture negative cows (Raizman, Fetrow and Wells, 2009), with a larger reduction in milk yield demonstrated in faecal culture positive cows compared to ELISA positive cattle (Gonda *et al.*, 2007). Furthermore, cattle with strong positive serum ELISA results had a significantly reduced 305-day milk yield and total lifetime production compared to serum ELISA negative, inconclusive or positive cattle (Lombard *et al.*, 2005), demonstrating the impact of stage of infection on milk production losses. Work estimating a positive association with previous lactation milk yield in infected animals prior to faecal MAP shedding found that monthly milk production decreased by 0.05kg to 1kg once shedding commenced, with a significant reduction in milk yield reported for cattle with faecal culture results of more than 30 CFU/gram of faeces (Smith *et al.*, 2009). Therefore, previous work finding a positive association between previous lactation milk yield and infection status may represent cohorts in earlier disease status, with higher yielding cattle suggested as genetically more susceptible to infection (Pritchard *et al.*, 2017), with work estimating no difference in milk yield between infected and non-infected animals possibly due to lack of accurate classification of infection status or equalisation of higher milk production from non-shedding infected animals and lower production from shedding infected animals which are normally high-producing, reducing significant difference between infected and non-infected groups (Johnson *et al.*, 2001; Smith *et al.*, 2009).

More recently, a systematic review and meta-analysis documented a reduced milk yield of 1.87kg per day in cattle with MAP detected in faeces by faecal culture or PCR, with between study variation observed mostly in studies defining a MAP infection based upon serology (McAloon *et al.*, 2016b).

b. Other production or disease impact

Slaughter weight and meat production

Weaning weight was 21.48kg less in calves born from suckler cows with strong positive antibody ELISA results versus calves born from antibody ELISA negative cows, with calf weight decreasing with increasing dam faecal MAP shedding (Bhattarai *et al.*, 2013). Economic losses of \$109.23 to \$156.60 per calf were estimated from moderate and heavy shedding dams (Bhattarai *et al.*, 2013). Calves inoculated with MAP up to 12 months of age demonstrated reduced bodyweight at 17 months, correlating to dose of MAP inoculation (Roy *et al.*, 2017). Weight loss of 31% and slaughter value reduction of 48% was also estimated for cows with positive MAP faecal culture compared to negative cattle (Kudahl and Nielsen, 2009). Mean slaughter weight of cull cows was reported to be 33.4kg less for every 10% increase in MAP herd seroprevalence, with an estimated revenue loss of \$1,150 annually (Johnson-Ifeorlundu, Kaneene and Lloyd, 1999).

Premature culling and mortality

Overall, MAP infected cattle are reported to be two times more likely to be culled prematurely than non-infected cattle, regardless of producer awareness of infection status (Lombard, 2011). Faecal culture negative cattle are significantly less likely to be culled in comparison with cattle with low faecal shedding or ELISA positive status (Smith *et al.*, 2010) or those that are categorised as faecal culture positive alone (Raizman, Fetrow and Wells, 2009), with main reasons for producer culling reported as decreased milk yield, mastitis, faecal shedding, positive serology and infertility in subclinical cases and faecal shedding, weight loss and reduced milk yield in clinical cases (Buergelt and Duncan, 1978).

A cross-sectional study in Michigan documented that herds classified as positive for paratuberculosis had a mortality rate that was 3% higher than negative herds (Johnson-Ifeorulundu, Kaneene and Lloyd, 1999), likely mainly representing the impact of producer culling. Costs of between \$1,607 and \$4,400 have been estimated per herd taking into account reduced slaughter weight and replacement costs, although this is likely inaccurate due to the age of the study (Johnson-Ifeorulundu, Kaneene and Lloyd, 1999). Increased on-farm mortality in positive cases has also been reported (Whittington *et al.*, 2019).

Predisposition to disease

Anecdotally, paratuberculosis has long been reported to increase susceptibility to other disease processes (McAloon *et al.*, 2019a), and increased risk of occurrence of clinical signs of paratuberculosis is also documented following parturition, bovine viral diarrhoea infection, parasite infestation and other causes of stress (Allen, Saba and Patterson, 1968; Hasonova and Pavlik, 2005). No association between SCC and paratuberculosis was reported in earlier work (Hendrick *et al.*, 2005; Lombard *et al.*, 2005; Gonda *et al.*, 2007), however there is a growing body of work estimating positive significant associations between SCC and MAP infection (McNab *et al.*, 1991; Baptista, Nielsen and Toft, 2008; Pritchard *et al.*, 2017; Martins *et al.*, 2018), with immunosuppression increasing susceptibility to infection hypothesised (Kreeger, Snider and Olcott, 1991; Kreeger and Snider, 1992). Increased incidence of clinical mastitis has also been reported in recent work (Pritchard *et al.*, 2017; Rossi *et al.*, 2017), with increased culling due to mastitis also documented in MAP positive herds (Merkal, Larsen and Booth, 1975; Arrazuría *et al.*, 2014).

Earlier work has estimated that MAP positive cows were 5 times more likely to be lame (Villarino and Jordan, 2005), with lameness reported as the most common disease present in MAP faecal positive cattle (Raizman *et al.*, 2007). In other more recent work, no difference in mobility score was observed between MAP antibody ELISA negative and positive cows, but MAP positive cattle had decreased lying time compared to MAP negative cattle at peak lactation (Charlton, Bleach and Rutter, 2019). However, it is hypothesised that this may be due to increased time standing eating secondary to negative energy balance associated with intestinal inflammation, reducing nutrient absorption (Charlton, Bleach and Rutter, 2019). Results from another recent study also suggest a temporal association between paratuberculosis infection and lameness; MAP antibody ELISA positive cows were 2.7 times more likely to become lame than their matched MAP negative controls and became lame an average of 3 months earlier, with strongly ELISA positive cattle 2.8 times

more likely to become lame after seroconversion than medium ELISA positive cattle (Smith and van Winden, 2019). Mobilisation of fat from the digital cushion during negative energy balance increasing risk of claw horn lesions is hypothesised, although causative lesions of lameness were not recorded in this study (Smith and van Winden, 2019).

There is little reported regarding paratuberculosis status and association with other diseases in cattle. Heavy shedding faecal culture positive cows were documented to be 3.6 times more likely to develop a DA than cattle with a negative faecal culture, with moderate shedding cattle 4.8 times more likely to develop clinical hypocalcaemia than negative counterparts (Raizman *et al.*, 2007). However, only five and two cattle with a DA and hypocalcaemia respectively were analysed in this work, with need for further investigation on greater numbers of cattle (Raizman *et al.*, 2007).

c. Fertility

It is well established that suboptimal fertility can contribute significant cost to the dairy herd (Cabrera, 2014), however associations between reproductive outcomes and subclinical paratuberculosis are less clear. There are a range of contradicting studies investigating the association between paratuberculosis and fertility, with many using different diagnostic methods such as milk and serum ELISA or faecal culture and PCR, making comparisons between studies more difficult. Similar to associations between the disease and other phenotypic traits, the impacts of stage of disease, lactation and criteria for MAP positive status assignment are contributory to the heterogeneity between available studies (Lombard *et al.*, 2005; Gonda *et al.*, 2007; Raizman *et al.*, 2007). However, misclassification bias is less likely in studies utilising an organism detection test to define MAP infection status such as faecal culture or PCR, compared to those using demonstration of an immune response to MAP (such as the antibody ELISA) (McKenna *et al.*, 2006).

It is hypothesised that the impact of subclinical MAP infection on fertility is derived from reduced gastrointestinal absorption and resultant negative energy balance (Johnson *et al.*, 2001), as observed in other disease processes (Fourichon, Seegers and Malher, 2000). Therefore, impacts upon fertility are more likely to be less noticeable in the earlier subclinical phase of disease (Hoogendam, Richardson and Mee, 2009).

A summary of current published studies investigating the association between reproductive outcomes and paratuberculosis is included in Table 1.

Table 1: Summary of studies investigating the association between reproductive outcomes and paratuberculosis

Studies demonstrating negative associations between reproductive outcomes and paratuberculosis			
Study title	Sample	MAP status definition(s)	Results
The effect of subclinical <i>Mycobacterium paratuberculosis</i> infection on days open in Michigan, USA, dairy cows (Johnson <i>et al.</i> , 2001)	533 cattle in 5 herds, USA	Serum ELISA positive vs serum ELISA negative Faecal culture positive vs faecal culture negative Serum ELISA positive and faecal culture positive vs all tests negative Serum ELISA positive and faecal culture negative vs all tests negative Serum ELISA negative and faecal culture positive vs all tests negative Serum ELISA positive or faecal culture positive vs all tests negative	Serum ELISA positive vs serum ELISA negative: 28 day increase in days open after parturition (standard error [SE]=11.4, $p<0.05$). No other significant associations with days open for all other status definitions.
The association between <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> faecal shedding or clinical Johne's disease and lactation performance on two Minnesota, USA dairy farms (Raizman <i>et al.</i> , 2007)	1052 cattle in 2 herds, USA	Faecal culture positive vs faecal culture negative Light/moderate/heavy faecal culture shedding vs faecal culture negative	Faecal culture positive vs negative: 2.8 times less likely to be inseminated (confidence interval [CI]= 1.4-5.7, $p<0.05$), 2.8 times less likely to conceive (CI= 1.4-5.7, $p<0.05$). No significant association with days open. Light faecal culture shedding vs negative: No significant difference in chance of insemination, 2.4 times less likely to conceive (CI= 1.1-5.5, $p<0.05$) Moderate faecal culture shedding vs negative: 3.1 times less likely to be inseminated (CI= 1.2-8.3, $p<0.05$), no significant difference in chance of conception. Heavy faecal culture shedding vs negative: 9.6 times less likely to be inseminated (CI= 2.6-24.5, $p<0.05$), 3.8 times less likely to conceive (CI= 2.2-5.3, $p<0.05$)
Fertility, udder health and milk	500 cattle in 1 herd,	Three positive milk antibody	Three positive milk antibody ELISA tests vs less than

production in cows that have high milk antibodies to paratuberculosis (Sibley, Orpin and Pearse, 2012)	UK	ELISA tests vs less than three positive milk antibody ELISA tests	three positive milk antibody ELISA tests: calving interval 416 days vs 386 days, only in the lactation the positive tests occur. Statistical significance of results is not stated in conference proceeding style communication.
The impact of paratuberculosis on milk production, fertility and culling in large commercial hungarian dairy herds (Ozsvari <i>et al.</i> , 2020)	4341 cattle in 5 herds, Hungary	Milk antibody ELISA positive vs milk antibody ELISA negative	Milk antibody ELISA positive vs milk antibody ELISA negative: 23.2 days additional calving to conception interval (95% CI: 9.2-37.3., $p<0.05$); 33.8 days additional calving interval (95% CI: 13.2-54.4, $p<0.05$); 0.42 additional services per conception (95% CI: 0.07-0.77, $p<0.05$); first service conception risk odds ratio of 0.49 (95% CI: 0.31-0.75. $p<0.05$).
Association of <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> infection with milk production and calving interval in Iranian Holsteins (Ansari-Lari, Haghighi and Mahmoodi, 2012)	252 cattle in 21 herds, Iran	Milk PCR positive vs milk PCR negative (animal level) Status retrospectively assigned from first lactation based upon milk PCR test results. At least one milk PCR positive cow per herd vs no milk PCR positive cows per herd (herd level)	Milk PCR positive vs milk PCR negative: no significant association with calving interval At least one milk PCR positive cow per herd vs no milk PCR positive cows per herd: 30 day increase in mean calving interval (SE: 21.84, $p=0.057$).
Effects of subclinical <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> infection on some physiological parameters, health status and production in dairy cows (Jurkovich <i>et al.</i> , 2016)	30 cows in 1 herd, Hungary	Faecal PCR positive vs faecal PCR negative	Faecal PCR positive vs faecal PCR negative: number of reproductive tract treatments = 5.7 (standard deviation [SD]: 4.3) vs 2.4 (SD: 1.9) $p<0.05$, number of inseminations= 2.8 (SD: 1.6) vs 1.4 (SD:0.5) $p<0.05$, calving interval= 437 days (SD: 66) vs 365 days (SD: 33) $p<0.05$, service period= 168.9 days (SD: 72.3) vs 84.6 days (SD: 33.1) $p<0.05$, no significant difference between categories for first service pregnancy rate.
Studies demonstrating positive associations between reproductive outcomes and paratuberculosis			
Study title	Sample	MAP status definition(s)	Results
Effect of <i>Mycobacterium</i>	4375 cattle in 232	Serum ELISA or faecal	Infected vs non-infected: 1.39% increase in pregnancy

<i>paratuberculosis</i> infection on production, reproduction, and health traits in US Holsteins (Gonda <i>et al.</i> , 2007)	herds, USA	culture positive (infected) vs serum ELISA negative and faecal culture negative (non-infected) Serum ELISA positive only vs serum ELISA negative only Faecal culture positive only vs faecal culture negative only	rate (SE= 0.67%. $p<0.05$). No significant difference in pregnancy rate found between serum ELISA positive and negative only status and faecal culture positive and negative only status.
Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows (Lombard <i>et al.</i> , 2005)	7879 cattle in 38 herds, USA	Serum ELISA inconclusive/positive/strong positive/negative	No significant difference between serum ELISA statuses and days non-pregnant except serum ELISA strong positive vs serum ELISA positive/negative: 161 days non-pregnant ($p<0.05$), compared to 206 days non-pregnant (serum ELISA positive) or 196 days non-pregnant (serum ELISA negative).
Higher non-return rate associated with <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> infection at early stage in Holstein dairy cows (Marcé <i>et al.</i> , 2009)	48914 cattle in 1069 herds, France	Serum ELISA positive (at least one test) vs serum ELISA negative in negative herd. Serum ELISA positive (at least one test) vs serum ELISA negative in positive herd. Status retrospectively assigned from first lactation based upon lifetime test results.	Serum ELISA positive vs serum ELISA negative in negative herd: 3.9% point increase in 200-day non-return rate overall (rate of no-return to oestrus in the 200 days following service) ($p<0.05$), 4.7% point increase of 200 day non-return rate for parity 1 (CI=2.1-6.8, $p<0.05$) and 2 (CI= 2-7.8, $p<0.05$) animals. No significant difference for parity 3 or more animals. Serum ELISA positive vs serum ELISA negative in positive herd: 2.4% point increase in 200-day non-return rate overall ($p<0.05$).
Effect of Johne's disease status on reproduction and culling in dairy cattle (Smith <i>et al.</i> , 2010)	2818 cattle in 6 herds, USA	Low positive-serum ELISA positive or faecal culture positive with less than 30 CFU/gram faeces or less than 50 CFU/tube vs serum ELISA and faecal culture	Low positive vs serum ELISA/faecal culture negative: proportional rates model effect estimate for time to calving (weighted for probability of culling and parity with interaction for test-positive status and parity >3) = 0.23 (SE= 0.12, $p<0.05$), demonstrating increased calving rate vs serum ELISA/faecal culture negative cattle, no

		negative High shedding-faecal culture positive with at least 30 CFU/gram faeces or at least 50 CFU/tube vs low positive category	significant effect estimate for time to first insemination. High shedding vs low positive: proportional rates model effect estimate for time to calving (weighted for probability of culling and parity with interaction for test-positive status and parity >3)= -1.07 (SE=0.50. $p<0.05$), demonstrating reduced calving rate vs low positive cattle. No significant effect estimate for time to first insemination.
Genetic variation in serological response to <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> and its association with performance in Irish Holstein-Friesian dairy cows (Berry <i>et al.</i> , 2010)	4789 cattle in 44 herds, Ireland	Serum antibody ELISA positive vs negative status Serum antibody ELISA sample to positive control ratio continuous value	In herds with serological MAP data: Serum antibody ELISA positive vs serum antibody ELISA negative & serum antibody ELISA sample to positive control ratio value: genetic correlation with calving interval (demonstrating genetic relationship between MAP status and calving interval) = -0.07 (SE: 0.337). In herds without serological MAP data: Serum antibody ELISA positive vs serum antibody ELISA negative: genetic correlation with calving interval (demonstrating genetic relationship between MAP status and calving interval) = parity 1: -0.61 (SE: 0.191). parity 2: -0.28 (SE: 0.196), parity 3: -0.14 (SE: 0.193) Serum antibody ELISA sample to positive control ratio: parity 1: -0.77 (SE: 0.218), parity 2: -0.51 (SE: 0.234), parity 3: -0.50 (SE: 0.227).
Studies demonstrating unclear or no associations between reproductive outcomes and paratuberculosis			
Study title	Sample	MAP status definition(s)	Results
Phenotypic effects of subclinical paratuberculosis (Johne's disease) in dairy cattle (Pritchard <i>et al.</i> , 2017)	58096 cattle in 2197 herds, UK	Low risk (all milk antibody ELISA tests negative or 1 test positive but last test negative) Medium risk (last test positive or at least 2 tests positive but not adjacently)	No significant difference in calving interval between different risk categories, for any parity. Days to first service: increased in high risk vs low risk cattle ($p<0.05$) only, in lactation 1 and combined lactation 1-3 model. Non return 56 days: medium risk vs low or high risk more likely to return to service after 56 days ($p<0.05$), in

		High risk (at least 2 adjacent tests positive, even if negative test follows) Status retrospectively assigned from first lactation based upon lifetime test results.	lactation 1 and combined model. Number of inseminations: increased in medium risk vs high risk in lactation 2 ($p < 0.05$), highest in medium risk vs high and low risk in lactation 3 and combined model.
Associations between dairy production indices and lipoarabinomannan enzyme-immunoassay results for paratuberculosis (McNab <i>et al.</i> , 1991)	14923 cattle in 304 herds, Canada	Serum lipoarabinomannan antigen ELISA test optical density result: herd mean average and cow level result.	Herd mean average serum lipoarabinomannan antigen ELISA test optical density result: no significant association between this and herd average calving interval. Cow level serum lipoarabinomannan antigen ELISA test optical density result: no significant association between this and calving interval.
Associations between reproductive performance and seropositivity for bovine leukemia virus, bovine viral-diarrhoea virus, <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> , and <i>Neospora caninum</i> in Canadian dairy cows (VanLeeuwen <i>et al.</i> , 2010)	2876 cattle in 151 herds, Canada	Up to 30 cattle randomly sampled per herd. Serum antibody ELISA positive vs serum antibody ELISA negative	Serum antibody ELISA positive vs serum antibody ELISA negative: no significant association with calving interval (days), no significant association with odds ratio of calving interval categories (>484 days. >534 days. >584 days), no significant association with odds ratio of first-service conception risk.
Paratuberculosis sero-status and milk production, SCC and calving interval in Irish dairy herds (Hoogendam, Richardson and Mee, 2009)	2602 cattle in 34 herds, Ireland	Serum antibody ELISA positive vs serum antibody ELISA negative (animal level) At least two serum antibody ELISA positive cows per herd vs less than two serum antibody ELISA positive cows per herd or vs no serum antibody ELISA positive cows in the herd	Serum antibody ELISA positive vs serum antibody ELISA negative: no significant association with calving interval. At least two serum antibody ELISA positive cows per herd vs less than two serum antibody ELISA positive cows per herd or vs no serum antibody ELISA positive cows in the herd: no significant association with calving interval.

		(herd level)	
Analysis of Johne's disease ELISA status and associated performance parameters in Irish dairy cows (Kennedy <i>et al.</i> , 2016)	3528 cattle in 22 herds, Ireland	Serum or milk antibody ELISA positive vs serum or milk antibody ELISA negative	Serum or milk antibody ELISA positive vs serum or milk antibody ELISA negative: no significant association with calving interval

Prospective, limited scale studies with controlled, detailed recording of events demonstrate mainly negative association between paratuberculosis and dairy cow fertility (Johnson-Ifeorlundu *et al.*, 2000; Raizman *et al.*, 2007); however larger scale epidemiological studies generally with cruder or more potentially biased outcome measures demonstrate either positive (Lombard *et al.*, 2005; Gonda *et al.*, 2007; Marcé *et al.*, 2009; Berry *et al.*, 2010; Smith *et al.*, 2010), unclear (Pritchard *et al.*, 2017) or no association (McNab *et al.*, 1991; Hoogendam, Richardson and Mee, 2009; VanLeeuwen *et al.*, 2010; Kennedy *et al.*, 2016) between paratuberculosis and fertility outcomes.

Current literature is also hampered by use of interval-based measures of fertility performance, presenting challenges for analysis of a progressive disease where infected animals are electively not served and are culled before the end of a lactation. Furthermore, an inability to link test positivity temporally to fertility indices in most studies, hinders accurate association of MAP status and fertility traits. Cumulatively, these aspects make the economic impact of paratuberculosis on fertility indices difficult to quantify.

d. Overall economic impact

Exact economic impact of paratuberculosis at herd level can be difficult to establish due to inaccuracy of prevalence estimates; the majority of infections are subclinical with variable productivity losses that are dependent on herd size, management and individual animal factors (Garcia and Shalloo, 2015). Worldwide, estimated economic losses due to paratuberculosis vary; in the USA, cost of disease has been estimated as \$100 per cow in MAP positive herds compared to negative herds, with a cost of \$200 per cow estimated in MAP positive herds with clinical cases (Ott, Wells and Wagner, 1999). A French estimate for the cost of a subclinical case with only reduced projected milk yield was €461 (Dufour, Pouillot and Durand, 2004), with an Irish study reporting a €168 to €253 reduction in profit margin per cow in MAP positive herds (Barrett *et al.*, 2006). A more recent study used an MCMC approach to estimate economic losses per cow within dairy herds of varying prevalence levels, by different region; it was calculated that 1% of total annual milk income was lost per MAP positive dairy herd overall, equating to \$198 million lost in the USA, \$75 million in Germany, \$56 million in France, \$54 million in New Zealand and between \$17 million and \$28 million in Canada (Rasmussen *et al.*, 2021).

UK cost estimates are limited and extremely variable; a £27 difference in expected net present value per dairy cow per annum has been estimated between an infected and disease free herd (Stott *et al.*, 2005), although this is likely to be higher given the increase in milk price and other costs since this work was published. Low cost estimates historically have provided less producer incentive for disease control, however there is a significant potential negative impact of paratuberculosis infection on society economic welfare (Barratt *et al.*, 2018). Furthermore, the economic impact of paratuberculosis may appear low or difficult to measure in very low prevalence herds, however will increase as proportion of subclinical cases increases over time if control measures are not employed (Tiwari *et al.*, 2008; Lombard, 2011).

1.8 Control of disease

a. Evidence-based control

There are no reports in literature of successful eradication of MAP bacteria from an infected cattle herd (Barkema *et al.*, 2018); control methods suggested have been mostly determined from knowledge surrounding MAP infection routes and host susceptibility, with long disease incubation and poor diagnostic test sensitivity contributing to a lack of successful field studies reporting risk factors for infection (McAloon *et al.*, 2019a). Existing risk assessment studies results have often disagreed with putative risk factors previously identified (McAloon *et al.*, 2015); misclassification of positive and negative herds due to poor diagnostic test accuracy and small sample sizes unable to surmount misclassification, alongside cross-sectional study designs with reduced ability to infer causality may be implicated in this (McAloon *et al.*, 2017b; McAloon *et al.*, 2019a). By design, many studies also struggled to evaluate individual control methods in isolation, with difficulties in proving a reduced incidence of new infections unless study periods were particularly elongated (McAloon *et al.*, 2019a).

Calving and colostrum management

Reducing neonatal calf contact with adult cow faeces was identified as the primary risk factor to control regarding MAP transmission in a recent systematic review of relevant literature (Doré *et al.*, 2012). Removal of calves within 2 hours of birth was documented to significantly reduce within herd apparent prevalence (Collins, Eggleston and Manning, 2010); when 90% of calves were removed within 15 minutes of calving herd risk of positivity was lowest, with herds being 2.3 times more likely to test positive when more than 50% of calves were removed within 30 minutes of birth versus within 15 minutes of birth (McAloon *et al.*, 2017b). Animals fed MAP negative colostrum and born to MAP negative dams were also 3.7 times more likely to test MAP positive if they spent more than 429 minutes in a calving pen of Wisconsin hygiene score greater than 2 (Nunney *et al.*, 2023). Furthermore, an increased herd risk of MAP positive milk filter by culture was significantly associated with overall reduced supervision at calvings (Cashman *et al.*, 2008). Critical limits of no more than 10% calves spending 60 minutes in the calving pen and no more than 5% cows scoring greater than 2 on a 3-point hygiene score scale has therefore been suggested to minimise neonatal exposure to MAP (McAloon *et al.*, 2015).

Segregation of ELISA positive and negative cows at calving, with cleaning and disinfection afterwards has been associated with reduced within herd apparent prevalence (Collins, Eggleston and Manning, 2010; Donat *et al.*, 2016), with herds housing sick or lame animals that may be more likely to be sub-clinically infected with MAP in the calving pen being 2.3 times more likely to test MAP positive (McAloon *et al.*, 2017b). Presence of zero sick, MAP positive or unknown risk cows housed in the calving pen is advised as a target (McAloon *et al.*, 2015).

Use of colostrum from individual MAP antibody ELISA negative cows following application of pre-milking teat disinfection and drying, was associated with reduced within herd apparent prevalence (Collins, Eggleston and Manning, 2010), with herds where more than 10% calves

were fed pooled colostrum being 2.1 times more likely to test positive (McAloon *et al.*, 2017b). Calves fed pasteurised colostrum compared to those fed raw colostrum also demonstrated a reduced early immune response to MAP as measured by interferon gamma (Stabel, 2008). However, this association is inconsistent in the literature and separating the effect of transmission from colostrum alone (without faecal contamination) can be challenging depending on study design (Nunney *et al.*, 2023). Other work reports no increased risk of positivity between calves fed MAP PCR positive colostrum and calves fed PCR negative colostrum (Pithua *et al.*, 2011), with no difference in proportion of cattle testing MAP positive, first or second lactation milk yield or survival, between cattle fed pasteurised or raw colostrum at birth (Godden *et al.*, 2015). Level of viable MAP shed in colostrum or milk in infected animals may be low depending on disease stage, potentially qualifying variation in associations found in literature (Stabel *et al.*, 2014), however control of faecal contamination of colostrum is still vital to control (Doré *et al.*, 2012), with this recognised as a significant risk during colostrum collection (Stewart *et al.*, 2005) and as such, a critical point for paratuberculosis control. A target of less than 10% samples with more than 100,000 CFU/ml total bacterial count and more than 10,000 CFU/ml coliform count has been suggested (McAloon *et al.*, 2015).

Youngstock management

Housing pre-weaned calves in groups during winter has been associated with increased herd level MAP seroprevalence (Tiwari *et al.*, 2009), with calves raised with future high MAP shedding cattle (more than 100 CFU MAP/g faeces) reported as 19.1 times more likely to be MAP infected (Benedictus *et al.*, 2008). In herds where calves were individually housed, the odds of a MAP positive milk filter culture were also significantly lower (Cashman *et al.*, 2008). However, further longitudinal work is required to characterise transmission rates and the impact of different control measures on this (Corbett *et al.*, 2019), with both longitudinal and cross-sectional study designs used in the above work, the latter providing little evidence for causation.

Exposure of calves to faeces of MAP shedding cattle (van Roermund *et al.*, 2007), particularly in the first six weeks of life, was associated with MAP infection in dairy herds (Obasanjo, Gröhn and Mohammed, 1997). Housing calves aged less than six months old with adult cattle also increased the risk of herd positivity (Diéguez *et al.*, 2008), alongside frequently grazing heifers on sick cow paddocks or slurry contaminated forage, although the latter risk only represented 1.3% variation in the Bayesian model (Norton, Heuer and Jackson, 2009; McAloon *et al.*, 2017b). However, although minimising contact with adult cattle manure until weaning contributed to reducing within herd apparent prevalence in one study (Collins, Eggleston and Manning, 2010), grazing or housing heifers near cows with direct slurry contact did not increase their risk of testing positive, with counterparts not in direct slurry contact at more risk of testing MAP positive in another study (McAloon *et al.*, 2017b).

Feeding pasteurised milk or calf milk replacer instead of raw milk, contributed to decreasing within herd paratuberculosis prevalence (Collins, Eggleston and Manning, 2010), with calves suckling foster cows two times more likely to test ELISA positive versus those fed calf milk

replacer (Nielsen, Bjerre and Toft, 2008). However more recently, both the pasteurisation process and calf milk replacer have been demonstrated to yield viable MAP bacteria (Grant *et al.*, 2002, 2017), leading to concern regarding common calf feeding methods. Calves fed calf milk replacer or copper treated or pasteurised milk did have significantly reduced numbers of viable MAP bacteria or no MAP bacteria in tissue versus those fed raw milk, however still faecally shed MAP bacteria, demonstrating milk feeding treatment alone was not effective to control MAP transmission (Steuer *et al.*, 2021). Waste milk feeding calves has been associated with both a higher MAP seroprevalence and increased clinical cases (Ridge, Baker and Hannah, 2005) and also a decreased risk of MAP transmission and possible protective effect (Ridge *et al.*, 2010). However, more recently, 51.5% of waste milk samples studied from 37 herds had detectable MAP on PCR testing (Leão *et al.*, 2017), reducing the viability of this feed option.

Adult cow management and culling

Herds with increased environmental hygiene scores or where manure was clearly visible were 1.3 to 1.7 times more likely to have a MAP positive environmental sample or test positive respectively, than those where only a trace amount of manure was recorded (Wolf *et al.*, 2016a; McAloon *et al.*, 2017b), representing poorer overall farm hygiene rather than increased rare infection of adult animals (Windsor and Whittington, 2010).

Larger herd sizes have long been demonstrated to be associated with increased risk of herds testing positive (Daniels *et al.*, 2002; Barrett *et al.*, 2011; McAloon *et al.*, 2017b), with herds increasing by 100 cows in size or purchasing more cattle being 2.1 and 1.31 times more likely to have a MAP positive environmental sample, respectively (Wolf *et al.*, 2016a). However, a lower risk of testing positive has also been documented for herds during periods of significant expansion (of more than 50%) (McAloon *et al.*, 2017b), with improved general management in larger herds or lower parity distribution reducing number of test positive animals suggested as contributory (Jago and Berry, 2011; McAloon *et al.*, 2017b).

The impact of testing and culling on MAP prevalence over time is less clear (McAloon *et al.*, 2019a). Early disease transmission simulation models with the ability to evaluate disease control measures individually initially demonstrated a clear consensus (Marcé *et al.*, 2010). Testing and culling adult cattle alone without managing routes of infection was demonstrated as ineffective in multiple studies (Weber, 2006; Kudahl, Nielsen and Østergaard, 2008), with combining strategies strongly recommended (Kudahl, Nielsen and Østergaard, 2011). Using repeated antibody ELISA testing alongside separate management or culling of high risk animals and control of other infection routes resulted in a simulated 10% prevalence reduction over 5 to 7 years (Kudahl, Nielsen and Østergaard, 2008). A recent French study using individual based models has also reported that both testing and culling and reducing calf exposure to adult cow faeces were required to prevent an increase in herd MAP prevalence, but that calf management was still the most powerful measure in achieving this (Camanes *et al.*, 2018). However, other work has demonstrated increased value of testing and culling alone, with risk based culling resulting in decreased prevalence over time but without complete disease eradication (Lu *et al.*, 2010; Al-Mamun *et al.*, 2017). It has been shown that this approach did prove more cost-effective than environmental

hygiene management in smaller herds (Kirkeby *et al.*, 2016; Smith, Al-Mamun and Gröhn, 2017). However, it is important to note that environmental hygiene control is a cornerstone in the management of many endemic diseases, with removal of these potentially resulting in significant negative impacts on animal health and welfare and therefore sustainability of the herd.

Vaccination

Vaccination implementation in sheep has demonstrated clinical success and significant cost benefit (Juste and Casal, 1993; Fridriksdottir *et al.*, 2000; Dhand *et al.*, 2013). A recent meta-analysis and further work reported that vaccinating cattle has a positive impact, with a delay in the onset of clinical signs and reduced MAP shedding (Bastida and Juste, 2011; Alonso-Hearn *et al.*, 2012), however prevention of infection has not been demonstrated (Kalis *et al.*, 2001). Commercial vaccines used currently are killed MAP whole cells suspended in an oil emulsion, with development of live attenuated vaccine candidates potentiating cell mediated and humoral immune response reported in recent literature (Faisal *et al.*, 2013; Bannantine and Talaat, 2015). Reduced uptake of vaccination has largely been attributable to interference of the vaccination with national tuberculosis eradication programs (Bastida and Juste, 2011), leading to vaccination banning in some countries (Garcia and Shalloo, 2015). The currently available inactivated vaccine reduces the sensitivity of the SICCT and the specificity of serological tests used to detect MAP (Coad *et al.*, 2013). However, modification of the diagnostic criteria and reagents used in the SICCT improved the capacity to distinguish *Mycobacterium bovis* infected animals, whether vaccinated for paratuberculosis or not (Serrano *et al.*, 2017).

Vaccination has been demonstrated to be more economically beneficial to dairy producers than testing and culling and improved hygiene management in disease transmission simulation studies (Cho *et al.*, 2012; Lu *et al.*, 2013), with regular bovine tuberculosis testing or frequent testing of small groups of cattle being the only scenarios in which a cost benefit was not simulated (Groenendaal *et al.*, 2015).

b. National control programs

World-wide

There are a variety of voluntary and compulsory programs in place world-wide (Nielsen, 2009). Australia, Canada, Denmark, the Netherlands, the UK and USA are examples of endemically infected countries with long standing active control programmes in place (Geraghty *et al.*, 2014). Structured RAMPs have been successfully adopted and maintained across such countries, with an initial risk assessment of farm management procedures and subsequent risk score assignment, with the risk assessment outcome being formation of a structured management plan and overall herd risk score (McAloon *et al.*, 2015). A reduction in the incidence of paratuberculosis was demonstrated following implementation of a standardised RAMP control plan in 8 USA dairy herds (Espejo *et al.*, 2012), although this has not been reported in larger scale studies (Sorge *et al.*, 2011b).

However, inter-observer variation represented 24% of the differences in RAMP score in one study (Pieper *et al.*, 2015), with end point testing of adult cattle or annual risk assessment comparison being often the only means of evaluating progress (McAloon *et al.*, 2015). An

alternative to end-point testing has been developed within the food production industry, with a risk based system using HACCP to ensure food safety at all stages of the food chain except primary production (Ropkins and Beck, 2000). Use of this system has been successfully developed for paratuberculosis control in Irish dairy herds, with use of evidence based critical control points, and suggested targets and critical limits for these (McAloon *et al.*, 2015). Unfortunately participation rate in voluntary paratuberculosis control programmes is commonly less than 30% (Nielsen, 2011), however, use of small, producer-led paratuberculosis control focus groups has resulted in more successful implementation of control measures (Roche *et al.*, 2015).

United Kingdom

Action Johne's, an industry initiative was set up by milk processor Dairy UK in 2009, as a voluntary education and engagement programme and more recently has developed a structured control programme for paratuberculosis management in the UK national dairy herd, mandatory for all Red Tractor farm assured dairy herds, representing 95% of all UK dairy farms (Orpin, Sibley and Bond, 2020).

There are six control strategies used for the programme; biosecurity protect and monitor for herds with confirmed disease freedom, improved farm management and/or test and cull for low prevalence herds, improved farm management and strategic testing, breed to terminal sire or firebreak vaccination for higher to very high prevalence herds (Orpin *et al.*, 2022):

Since the commencement of the plan, 70% of participating herds use milk antibody ELISA tests with 42% of herds embarked upon a quarterly testing programme (Orpin *et al.*, 2022), although culling decision making and practicalities segregating cows were highlighted as key areas of uncertainty for farmers, demonstrating the value of veterinary involvement in maintaining farmer engagement (Orpin, 2017).

1.9 Conclusions and study aims

Paratuberculosis clearly represents a significant challenge to worldwide cattle production; endemic prevalence exists in areas of widespread ruminant farming, with the fatal disease characterised by significant production losses and welfare impacts on both livestock and farmers. Extensive environmental and post-pasteurisation survival alongside potential association with incurable Crohn's disease in humans further magnify relevance of the disease in the food producing sector. Successful control is impeded by lack of accurate available diagnostic testing to identify infected and infectious cattle, in a disease with a prolonged latent period.

Difficulty with identification of infected animals and only recent uptake of national testing programmes has limited literature estimating the level of production loss associated with infection. Given the equivocal existing evidence on association between fertility and MAP test status and potential significant economic implications, further research into the effect of paratuberculosis on reproductive efficiency is warranted.

The aim of this study was to help provide clearer evidence on the exact associations between fertility performance and positive MAP test status (measured by ELISA) reported in existing literature, using a large sample of commercial dairy herds from the United Kingdom. Associations between timing of non-negative MAP test statuses and risk of insemination and conception were assessed using two different models, to determine the contribution of each of these factors to associations between MAP test status and overall fertility performance. A third model represented a culmination of associations between MAP test status and insemination and conception risk.

Chapter 2: Materials and methods

2.1 Data collection and restructuring

a. Ethical approval

The study protocol was approved by the University of Nottingham School of Veterinary Medicine and Science Ethical Review Committee (Approval number, 2838 190927; Approval date, 28 August 2019).

b. Data collection

Longitudinal milk recording, fertility and MAP antibody ELISA milk test data were collated from 93 UK dairy herds from the database of a national milk recording organisation (Quality Milk Management Services Ltd). No data regarding herd management practices were available. Data were anonymised and converted into a consistent database format. The initial dataset consisted of data from 325,707 lactations in 95,823 cows, beginning in the years 1978 to 2020.

c. Diagnostic testing

Milk samples were tested for MAP antibody via the commercial MAP IDEXX milk antibody ELISA (*Mycobacterium paratuberculosis* Antibody Test Kit, IDEXX Europe (Fry, Kruze and Collins, 2008)). This assay is an indirect antibody ELISA (Figure 5); the target antigen is coated on the solid phase plate, to which any antibodies when added within the milk sample will bind to. An enzyme-labelled antibody conjugate specific to these antibodies is then added, with the subsequent addition of the conjugate substrate initiating a colour to develop in the wells where antibody is detected. Degree of colour development is proportional to the amount of sample antibody bound to the solid phase antigen and is quantified by measuring optical density of the final sample using an ELISA plate reader. Results are expressed as a ratio of the optical density of the sample to that of the positive control. Both the positive and negative controls within the test kit used remained consistent over the sampling period.

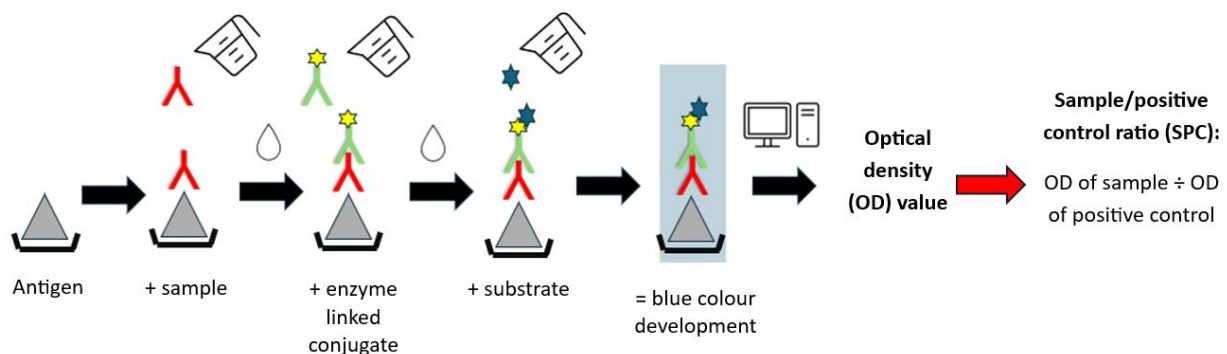


Figure 5: Indirect antibody milk ELISA assay steps.

The sensitivity of the antibody ELISA assay used in this study was low at 30.2% (95% CI: 18.3-44.3%) , although specificity was very high at 99.71% (95% CI: 98.4-99.99%) (Fry, Kruze and Collins, 2008). Classification of MAP milk antibody ELISA test results and categorisation into risk-based statuses based upon testing history are defined in Table 2. The closest MAP test

status was allocated to each risk period or insemination, if there was no MAP test status within 180 days of the end of the risk period or insemination, the MAP test status for this was coded as missing. Additional binary variables were constructed for each risk period and insemination, representing the cow moving from a negative to a non-negative MAP test status within various timeframes shown in Table 7, following the risk period or insemination.

Table 2: Definition of *Mycobacterium* subspecies *avium* *paratuberculosis* (MAP) test statuses.

MAP test status	Definition
Currently negative	All tests negative (antibody titre less than sample-to-positive control ratio of 0.2) or previously tested Uncertain/Provisionally Positive once but have subsequently tested negative on three consecutive occasions
Uncertain	Antibody titre is equal to or more than the sample-to-positive control ratio of 0.2 and less than 0.3 and/or cows that have previously tested Positive (antibody titre equal to or more than sample-to-positive control ratio of 0.3) but have since tested negative on less than three consecutive occasions
Provisionally Positive	The latest MAP ELISA milk test is Positive
Positive	Two MAP ELISA milk test Positives out of the last three tests; will not be re-classified once categorised as Positive, regardless of subsequent MAP ELISA results

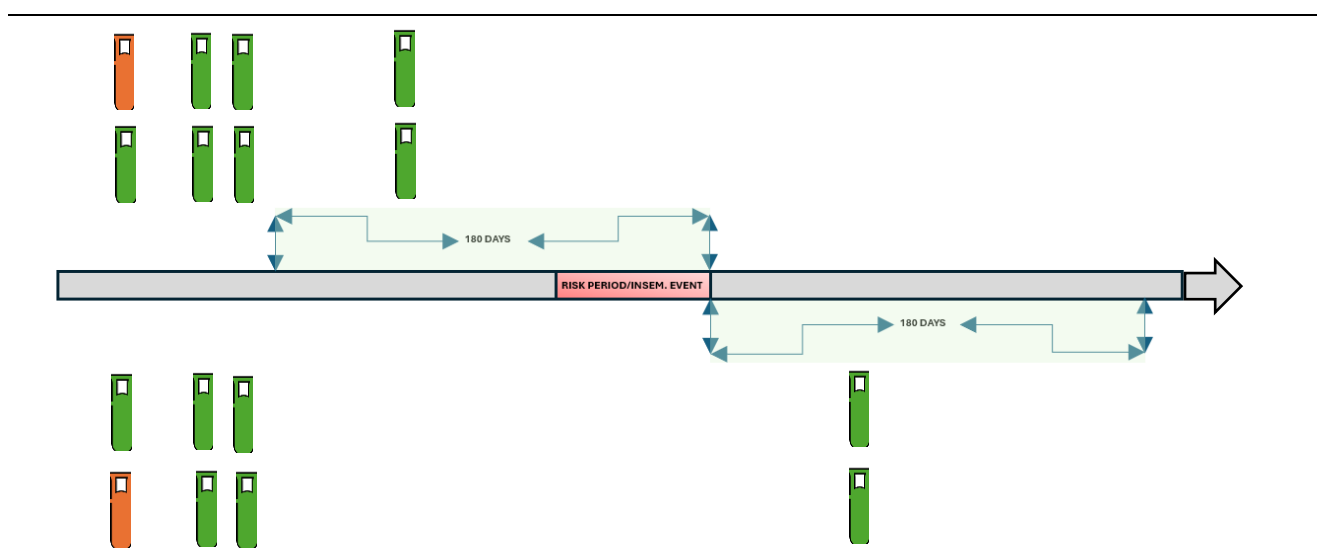
MAP, *Mycobacterium* subspecies *avium* *paratuberculosis*; ELISA, enzyme-linked immunosorbent assay.

The graphical representation shown in Figure 6a demonstrates how current MAP test statuses and their defining historical MAP antibody ELISA results were associated temporally with the 21-day risk periods or inseminations they were allocated to for further analysis. Illustration of the allocation of previous lactation MAP test status and future non-negative MAP test status to 21- day risk periods or inseminations, is shown in Figure 6b and 6c, respectively.

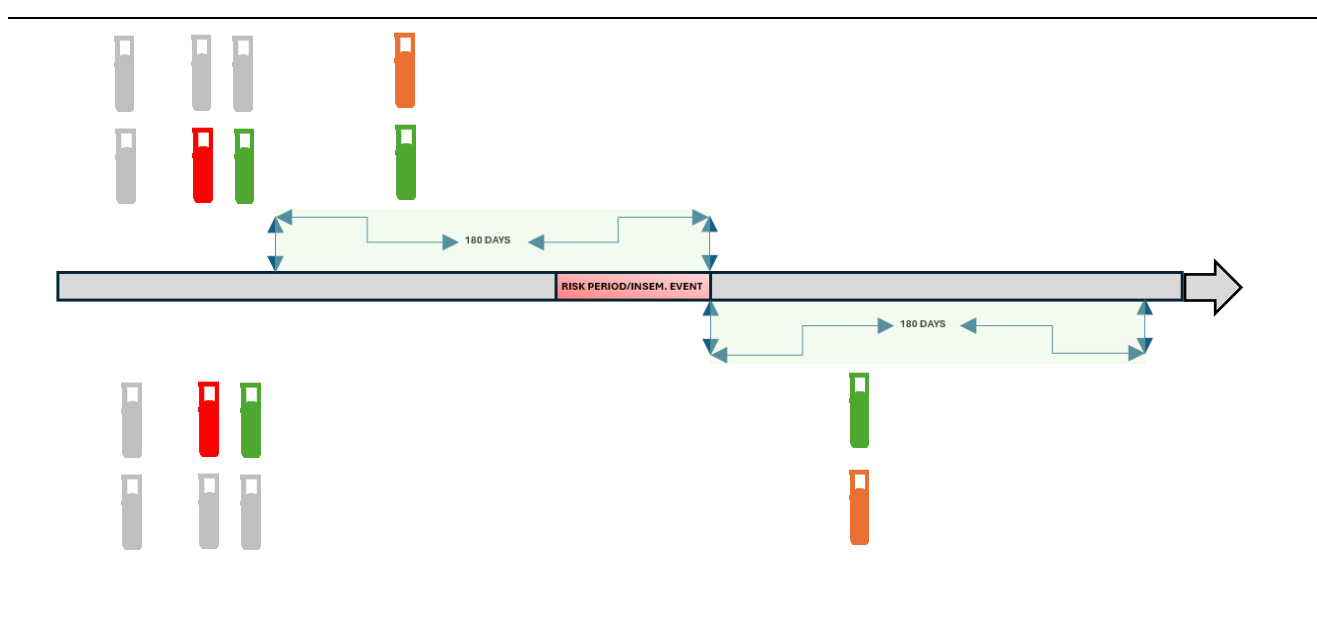
Figure interpretation

ELISA antibody test	Sample-positive-control ratio	ELISA antibody test	Sample-positive-control ratio
	<0.2		≥ 0.3
	$0.2 - <0.3$		Not relevant

Currently negative MAP test status



Uncertain MAP test status



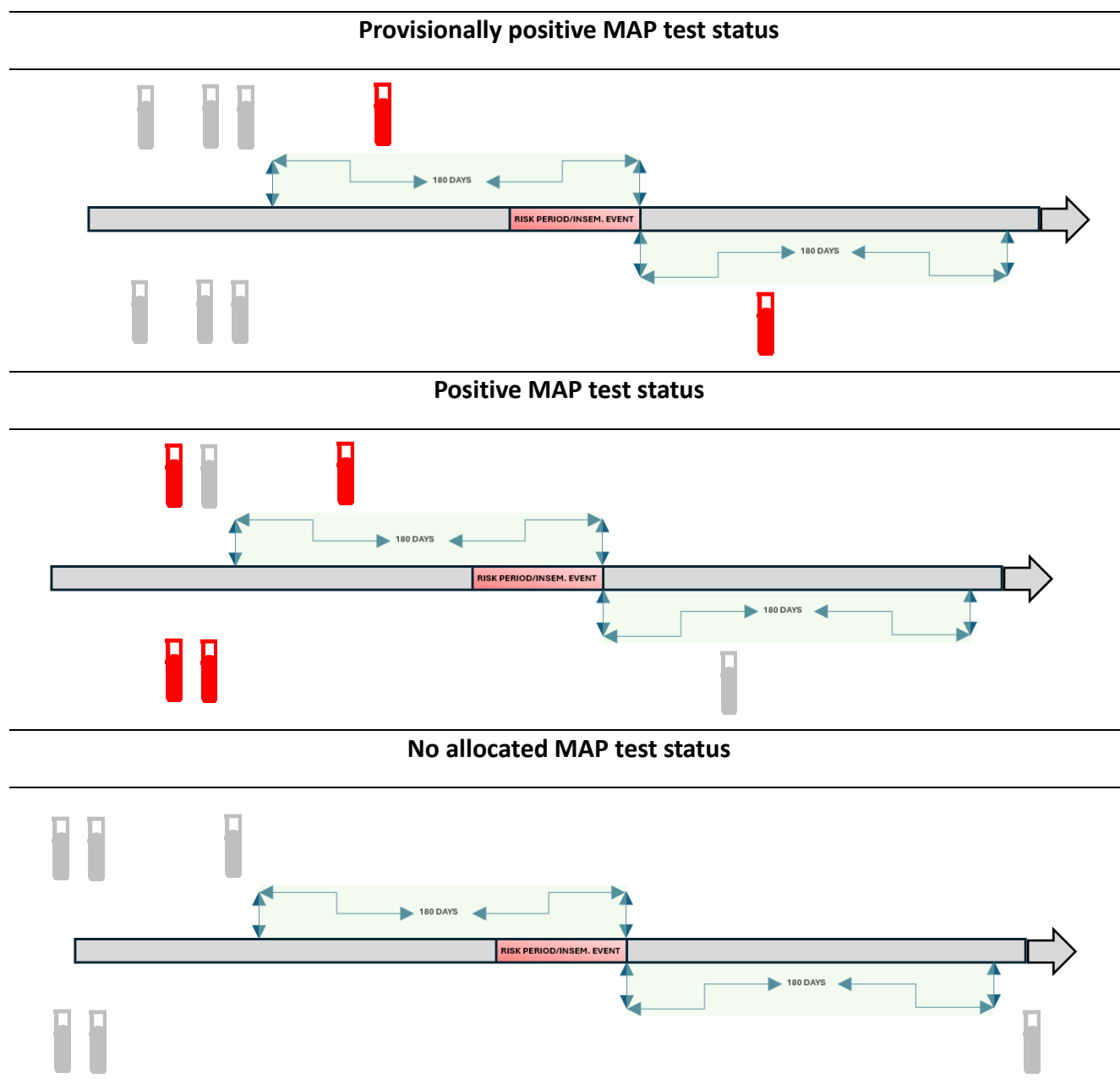


Figure 6a: Current MAP test status and relevant historical MAP milk antibody result allocation to 21-day risk period or insemination.

Figure interpretation

ELISA antibody test	Sample-positive-control ratio	ELISA antibody test	Sample-positive-control ratio
	<0.2		≥ 0.3
	$0.2 - <0.3$		Not relevant

Previous lactation status

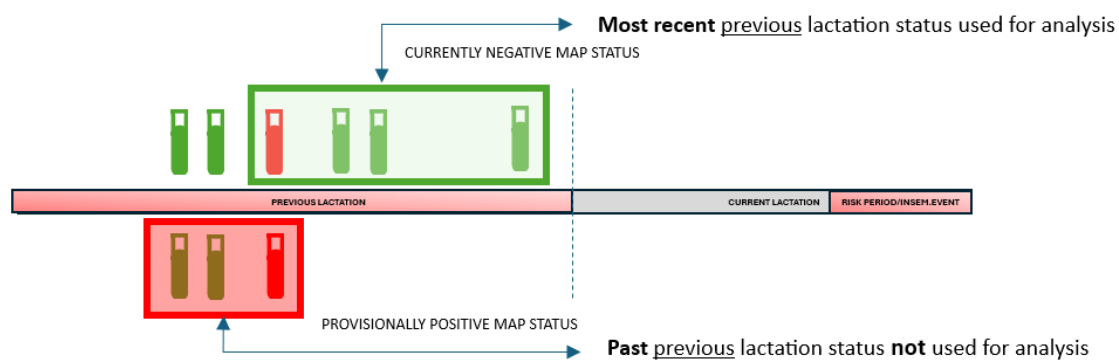


Figure 6b: Previous lactation MAP test status and relevant historical MAP milk antibody result allocation to 21-day risk period or insemination.

Figure interpretation

ELISA antibody test	Sample-positive-control ratio	ELISA antibody test	Sample-positive-control ratio
	<0.2		≥ 0.3
	0.2 - <0.3		Not relevant

Transition to future non-negative status

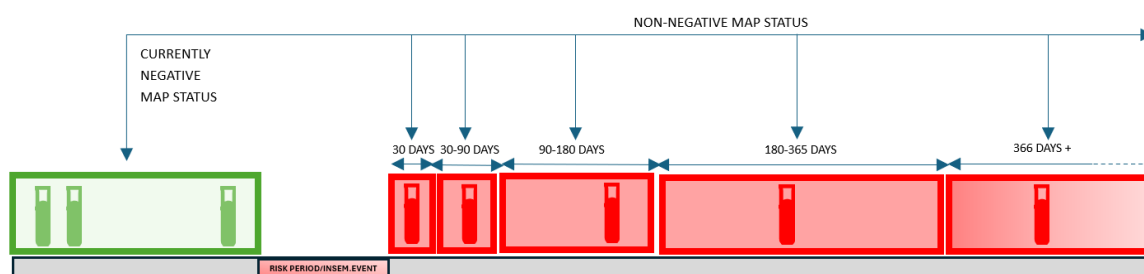


Figure 6c: Future non-negative status and relevant historical and future MAP milk antibody result allocation to 21-day risk period or insemination.

d. Data quality screening

Novel data quality assessment criteria were developed and applied to each data set. Each dataset was initially screened for duplicate event records (calving, insemination, pregnancy diagnosis, dry off and MAP antibody ELISA test data). Further assessment criteria were applied at herd-year level, facilitating removal of herds with missing event data or physiologically implausible data, considered to have potential to impact results (Figure. 7). These included measurement of the proportion of lactations with an unrelated insemination per calving and a physiologically plausible calving interval, alongside measurement of proportion of inseminations with unresolved outcomes. Apparent conception rate was analysed to detect systematic under-recording of herd insemination data. Lactation level criteria were also applied and lactations were only retained with a calving event that occurred between 01/01/2010 and 30/09/2018. Basic statistics describing the datasets before and after data quality assessment are included in Tables 3 and 4. Following screening of data, 121,762 lactations including 266,246 inseminations in 51,345 cows from 78 herds were retained for further analysis. More detailed basic statistics describing these herds are provided in Tables 5 and 6.

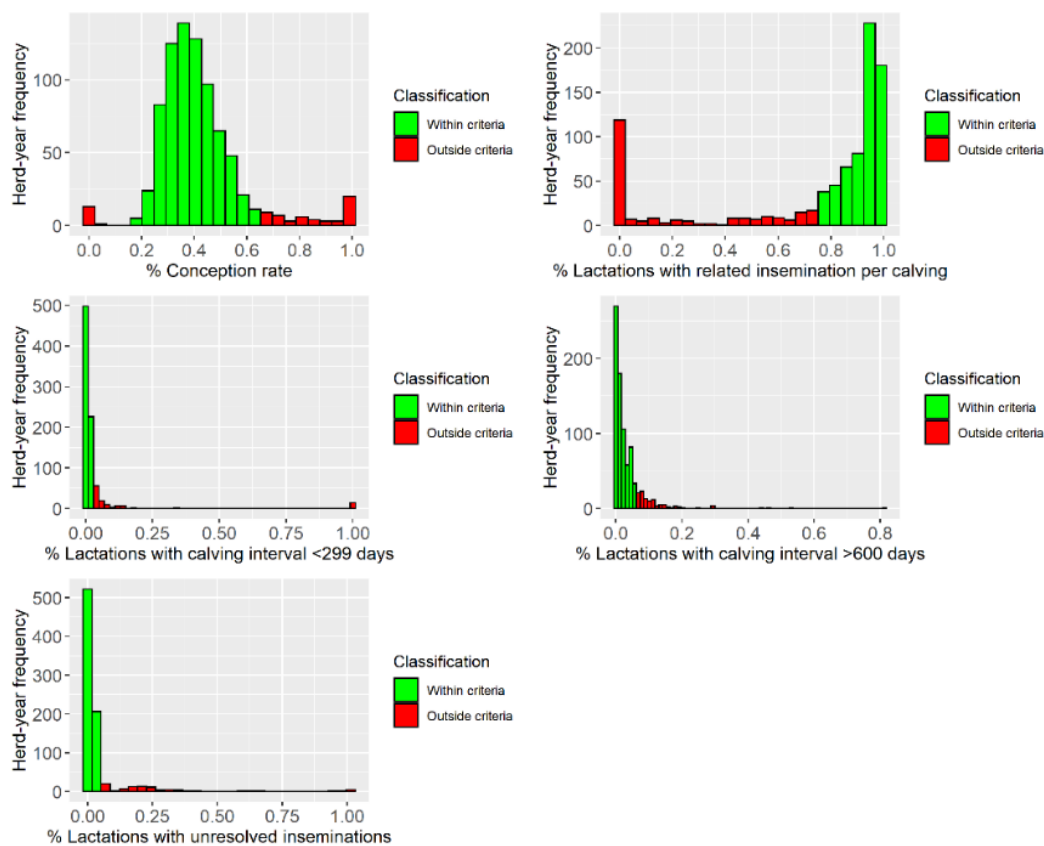


Figure 7: Distribution of data quality measures across herd-years; the proportion of lactations with an unrelated insemination per calving and a physiologically plausible calving interval and the proportion of inseminations with unresolved and successful outcomes.

Table 3: Summary of basic production and fertility metrics for 93 herds in the initial dataset and for 78 remaining herds used in model building following data quality screening ^a.

Parameter	Before data quality assessment:				After data quality assessment:			
	Mean	Min	Max	Median	Mean	Min	Max	Median
Herd size	162	1	1,310	120	299	53	1,292	229
Predicted 305-day milk yield (kg)	8,502	3,290	13,522	8,557	8,563	4,421	11,832	8,707
Culling rate (%/year)	13.95	0	100	14.42	23.75	0	44.02	24.56
Calving interval (days)	449	1	18,288	395	398	365	433	398
Conception rate (%)	43.91	0	100	41.21	39.77	26.33	63.21	38.57

^aThe mean value was calculated for each herd over the time period that herd featured in the data set; the values in the table describe the distribution of these herd values.

Table 4: Summary of number of *Mycobacterium* subspecies *avium* *paratuberculosis* (MAP) antibody milk enzyme-linked immunosorbent assay (ELISA) tests in initial dataset of 93 herds and for 78 remaining herds used in model building following data quality screening ^a.

	Before data quality assessment: Number of MAP antibody ELISA tests				After data quality assessment: Number of MAP antibody ELISA tests			
	Currently negative	Uncertain	Provisionally positive	Positive	Currently negative	Uncertain	Provisionally positive	Positive
Lactation 1	111,781	3,115	1,935	442	60,285	1,578	873	203
Lactation 2	87,227	4,188	2,171	1,218	47,599	2,086	1,081	581
Lactation 3	58,928	3,863	1,663	1,384	31,594	1,916	795	611
Lactation 4	37,095	2,829	1,187	1,247	19,759	1,347	573	584
Lactation >4	46,849	4,200	1,471	1,823	23,938	1,858	686	791

MAP, *Mycobacterium* subspecies *avium* *paratuberculosis*; ELISA, enzyme-linked immunosorbent assay.

^aGrouped by MAP test status and parity; definitions for MAP test status provided in Table 2.

Table 5: Summary of production and fertility metrics and *Mycobacterium* subspecies *avium* *paratuberculosis* (MAP) antibody milk enzyme-linked immunosorbent assay (ELISA) testing characteristics for 78 herds used in model building^a.

Parameter	Mean	Min	Max	25% quantile	50% quantile	75% quantile
Herd size	299	53	1292	141	229	363
Predicted 305-day milk yield (kg)	8,563	4,421	11,832	7,373	8,707	9,731
Culling rate (%/year)	23.75	0	44.02	19.37	24.56	29.46
Calving interval (days)	398	365	433	391	398	408
Conception rate (%)	39.77	26.33	63.21	33.63	38.57	44.62
21-day pregnancy rate (%)	13.74	8.46	21.71	11.81	13.50	15.01
21-day submission rate (%)	33.93	21.66	57.15	28.81	33.93	37.40
Days to first insemination (days)	74	56	97	69	73	79
Days to conception (days)	118	87	154	110	119	126
MAP antibody milk ELISA tests per lactation	1.72	0	5.74	0.80	1.82	2.59
Lactations with >1 MAP antibody milk ELISA test (%)	53.88	0	100	8.54	53.88	86.86
Lactations with >2 MAP antibody milk ELISA tests (%)	31.42	0	95.58	0.61	22.71	59.25
Days between MAP antibody milk ELISA	132	33	609	97	116	146

MAP, *Mycobacterium* subspecies *avium* *paratuberculosis*; ELISA, enzyme-linked immunosorbent assay.

^aThe mean value was calculated for each herd over the time period that herd featured in the data set; the values in the table describe the distribution of these herd values. A summary of key fertility metrics across different current MAP test statuses is included in Chapter 3.

Table 6: Summary of number of *Mycobacterium* subspecies *avium* *paratuberculosis* (MAP) antibody milk enzyme-linked immunosorbent assay (ELISA) tests in data set^a

	MAP test status	Lactation 1	Lactation 2	Lactation 3	Lactation 4	>Lactation 4
Number of MAP milk ELISA tests	Currently negative	60,285	47,599	31,594	19,759	23,938
	Uncertain	1,578	2,086	1,916	1,347	1,858
	Provisionally Positive	873	1,081	795	573	686
	Positive	203	581	611	584	791
	Transition to Non-Negative from Currently Negative MAP test status ^b	293	423	340	214	235
Number of cows						

MAP, *Mycobacterium* subspecies *avium* *paratuberculosis*; ELISA, enzyme-linked immunosorbent assay.

^aGrouped by MAP test status and parity, and number of cows transitioning from Currently Negative to Non-Negative MAP test status, across different parities.

^bUncertain/Provisionally Positive/Positive MAP test status

e. Data restructuring

Fertility and MAP milk antibody ELISA test data were amalgamated and two datasets constructed. Restructuring was carried out in R version 4.0.3 (R Core Team, 2020) using the tidyverse package (Wickham *et al.*, 2019). Dataset 1 was restructured into a format where each unit of data represented a 21-day risk period in every lactation between 21 and 336 days-in-milk (DIM) to evaluate the association between MAP test status and risk of insemination and pregnancy occurring per risk period. Dataset 2 was restructured such that insemination events were the units of data to evaluate association between MAP test status and risk of conception occurring for a given insemination. Occurrence of insemination and pregnancy in each 21-day risk period and occurrence of pregnancy for each insemination were included as binary variables based upon recorded events; if no pregnancy diagnosis was recorded then pregnancy was determined to have occurred where a calving was recorded 257 to 307 days after an insemination (Hudson *et al.*, 2012). In the case of multiple successive inseminations fulfilling this criteria (7.96% lactations), the closest insemination to average gestation length for Holstein breed cattle was considered the successful one (Norman *et al.*, 2009). Risk periods were censored from analysis when cows were no longer eligible for insemination (i.e. if pregnant or marked not to breed). For each risk period and insemination, multiple potential explanatory variables were also calculated, listed in Table 7.

Table 7: Variables and variable type calculated at each level of data per 21-day risk period or insemination in datasets 1 and 2 respectively.

Variable	Variable type
21-day risk period or insemination level	
Inseminated ^a	Binary (served or not served)
Insemination outcome	Binary (becomes pregnant or does not)
DIM at start of risk period/at insemination	Continuous
Month of insemination/risk period	Categorical; January, February, March, April, May, June, July, August, September, October, November, December
Year of insemination/risk period	Categorical; 2010-2019
ISI ^b	Continuous
ISI category ^b	Categorical; <19 days, 19-26 days, 27-37 days, 38-52 days, >52 days, none ~ (first insemination, missing)
Insemination number ^b	Categorical; 1,2,3,4 or >4
MAP test status	Categorical; Currently negative, Uncertain, Provisionally Positive, Positive, No status – (missing)
Currently Negative to non-negative MAP test status within next 30 days	Binary indicator representing increase from Currently Negative to non-negative MAP test status within 30 days after end of risk period/insemination or not
Currently Negative to non-negative MAP test status within next 30 to 90 days	Binary indicator representing increase from Currently Negative to non-negative MAP test status within 30 to 90 days after end of risk period/insemination or not
Currently Negative to non-negative MAP test status within next 90 to 180 days	Binary indicator representing increase from Currently Negative to non-negative MAP test status within 90 to 180 days after end of risk period/insemination or not
Currently Negative to non-negative MAP test status within next 180 to 365 days	Binary indicator representing increase from Currently Negative to non-negative MAP test status within 30 days after end of risk period/insemination or not
Currently Negative to non-negative MAP test status after more than 365 days	Binary indicator representing increase from Currently Negative to non-negative MAP test status within 365 days after end of risk period/insemination or not
Lactation level	
Month in which lactation began	Categorical; January, February, March, April, May, June, July, August, September, October, November, December
Year in which lactation began	Categorical; 2010-2018
Parity of cow	Categorical (1,2,3,4, or >4)
Previous lactation MAP test status	Categorical; Currently negative, Uncertain, Provisionally Positive, Positive, No status- (missing)
305-day milk yield (x 1,000 kg)	Continuous

ISI, Interservice Interval; MAP, *Mycobacterium* subspecies *avium* *paratuberculosis*

^aApplicable to 21-day risk period level only (dataset 1).

^bApplicable to insemination level only (dataset 2).

2.2 Statistical analysis

a. Models

Model 1

A logistic regression model with the binary event of pregnancy becoming established as the outcome variable was used to evaluate the association between the probability of a cow becoming pregnant in a given 21-day risk period, MAP test status and other potential explanatory variables. A multilevel hierarchical model was used with a three-level structure to account for correlations within the data, with risk periods nested within cows nested within herds. Data from dataset 1 were used to construct the model and the model specification took the form:

$$Pregnancy_{tij} \sim \text{Bernoulli}(\text{mean} = \mu_{tij})$$

$$\ln\left(\frac{\mu_{tij}}{1-\mu_{tij}}\right) = \beta_0 + \boldsymbol{\beta}\boldsymbol{\chi}_{tij} + \nu_{0j} + \nu_{0ij}$$

$$\nu_{0j} \sim N(0, \sigma_{\nu_0}^2)$$

$$\nu_{0ij} \sim N(0, \sigma_{\nu_0}^2)$$

where $Pregnancy_{tij}$ is whether the i th cow in the j th herd becomes pregnant during risk period t ; μ_{tij} is the fitted probability of $Pregnancy_{tij}$, β_0 is the regression intercept, $\boldsymbol{\beta}$ is the vector of coefficients for the vector of explanatory variables $\boldsymbol{\chi}$; ν_{0j} and ν_{0ij} are the random effects to represent herd and cow level variation respectively, with $\sigma_{\nu_0}^2$ and $\sigma_{\nu_0}^2$ the variances of the normal distributions of the respective random effects terms.

Model building was carried out using the lme4 package (Bates *et al.*, 2015) in R, using iterative generalized least squares for initial parameter estimation. Initial model building was via stepwise forward selection, with non-MAP test status explanatory variables (*i.e.* lactation number, DIM, 305-day yield) added before MAP test status variables. Explanatory variables were retained in the model if the 95% confidence interval for their coefficients did not cover zero and if rejected, were reintroduced into the model after initial variable selection and retained if they satisfied the criteria described above. Where one or more categories of a categorical variable were significant, all categories for that variable were retained. First order interactions between explanatory variables were only tested if considered clinically important and polynomial functions tested for all continuous variables up to power three. Interactions and polynomial functions were retained where the 95% confidence interval for their coefficients did not cover zero. Shapes described by polynomial functions were also compared to patterns in raw data to ensure appropriate shapes were fitted. Final parameter estimates were generated with MCMC using the brms package (Bürkner, 2017, 2018, 2021) using 2 chains and 2,000 iterations per chain after a 1,000 iteration burn in. Initial parameter values for chains were randomly generated from within appropriate parameter space. The Gelman-Rubin statistic was used to evaluate chain convergence, with values close to 1 indicating satisfactory convergence. MCMC was used for final parameter estimation due to increased likely reliability of estimates (Browne and

Draper, 2006) alongside provision of model fit assessment via generation of full posterior predictions.

Model 2

To evaluate the association between the probability of a cow being inseminated in a given 21-day risk period, MAP test status and other potential explanatory variables, another logistic regression model was constructed with the binary event of occurrence of insemination as the outcome variable. The same dataset 1, model specification and building process was used to construct this model as Model 1.

Model 3

To assess the association between the probability of a cow becoming pregnant as a result of a given insemination, MAP test status and other potential explanatory variables, a third logistic regression model was constructed with the binary event of insemination success as the outcome variable. Data from dataset 2 were used to construct this model, model building was carried out as previously described and the model specification took the form:

$$Conception_{ijk} \sim \text{Bernoulli}(\text{mean} = \mu_{ijk})$$

$$\ln\left(\frac{\mu_{ijk}}{1-\mu_{ijk}}\right) = \beta_0 + \boldsymbol{\beta}\boldsymbol{x}_{ijk} + v_{0k} + v_{0jk}$$

$$v_{0k} \sim N(0, \sigma_{v0}^2)$$

$$v_{0jk} \sim N(0, \sigma_{v0}^2)$$

where $Conception_{ijk}$ is whether the i th insemination in the j th cow in the k th herd resulted in a pregnancy; μ_{ijk} is the fitted probability of $Conception_{ijk}$, β_0 is the regression intercept, $\boldsymbol{\beta}$ is the vector of coefficients for the vector of explanatory variables $\boldsymbol{x}$; v_{0k} and v_{0jk} are the random effects to represent herd and cow level variation respectively, with σ_{v0}^2 and σ_{v0}^2 the variances of the normal distributions of the respective random effects terms.

b. Model assumption checking and assessment of model fit

Posterior predictive assessment was used to evaluate model fit (Green et al., 2009) and cross-validation used to check for model overfitting.

For each model, full posterior predictions were performed using the brms package (Bürkner, 2017, 2018, 2021), such that a predicted outcome was generated for each unit of data at each iteration of the MCMC chains. The data was then subset into a variety of samples in turn (e.g. based on variables used and not used in final models, or on random sampling); for each subset the 95% coverage interval of the predicted outcomes across MCMC chain iterations was compared with observed outcomes. Where the observed mean outcome was outside the 95% coverage interval for the predicted mean, steps were taken to improve model fit in this area; for example polynomial continuous representations of DIM in the risk period based models (Models 1 and 2) resulted in unsatisfactory fit and were replaced with categorical representation.

Cross validation was performed using the tidymodels package (Kuhn and Wickham, 2020). Data were split randomly (stratified on outcomes) into a training dataset consisting of 75%

of the data and a test dataset. Models based on the variables identified above were built using the test dataset, then predictions from these models generated for the test dataset. Ten-fold cross validation (Kohavi, 1995) was also performed. AUROC was calculated for each sampling method (within-and-out-of-sample predictions, and 10-fold cross validation). For each model, AUROC was less than 5% different across sampling methods, suggesting models were not substantially overparameterized.

c. Posterior predicted relative risks

Full posterior predictions were generated using each model as previously described. Predicted probability of insemination and pregnancy in a 21-day risk period and conception for a given insemination was calculated over 2,000 iterations for every category of current and future MAP antibody ELISA status variables whilst values of all other explanatory variables were set at the population mean to evaluate the impact of MAP antibody ELISA status variables alone. The predicted relative risk was calculated by division of sum of predicted outcomes where MAP status was set as non-negative by sum of predicted outcomes where MAP status was set as negative. The distributions of predicted relative risks were illustrated for each model per MAP status variable across the 2,000 iterations as median and 95% credible intervals.

Chapter 3: Results

3.1 Model estimates and posterior predictions

a. Model 1

Assessing the total of 365,142 21-day risk periods under analysis after excluding risk periods with missing 305-yield data, mean probability of pregnancy occurring in a given risk period was 0.140. Mean probability of pregnancy occurring in a given risk period for a cow with a Currently Negative MAP antibody ELISA status was 0.155; a cow with Uncertain status had a mean probability of pregnancy of 0.126 in a given risk period, with 0.125 and 0.066 mean probabilities of pregnancy in a given risk period for Provisionally Positive and Positive statuses respectively.

Parameter and variance estimates for model 1 are shown in Table 8; estimates for odds ratios were calculated by exponentiation of coefficient estimates. Herd level variance was estimated as 0.26 (0.025 standard error) and cow level variance estimated as 0.39 (0.016 standard error). Parity, 305-day yield and month of 21-day risk period and calving were explanatory variables included in final analysis; all were significantly associated with the probability of a cow becoming pregnant during a risk period.

All current and previous lactation non-negative MAP antibody ELISA statuses were negatively associated with probability of pregnancy occurring in a given 21-day risk period compared to Currently Negative status, except previous lactation Uncertain status. Magnitude of negative association was largest and significant in MAP test statuses defined by the highest number of recent Positive MAP antibody ELISA results (largest reduction in odds of cow becoming pregnant in a risk period associated with Positive status; Odds Ratio [OR], 0.64; 95% Credible Interval [CI], 0.52 - 0.79); this pattern was mirrored with Provisionally Positive and Positive previous lactation MAP antibody ELISA status significantly negatively associated with probability of pregnancy occurring in a given risk period. Transition from Currently Negative MAP antibody ELISA status to any non-negative MAP antibody ELISA status in the next 30 to 90 days after the end of a risk period was significantly negatively associated with probability of pregnancy occurring (OR, 0.64; CI, 0.53 - 0.79); transition from Negative to Positive MAP test status in the next 90 to 180 days (OR, 1.46; CI, 1.26-1.68), 180 to 365 days (OR, 1.72; CI, 1.36-2.12) and more than 365 days after the end of a risk period (OR, 2.14; CI, 1.27-3.53) was significantly positively associated with probability of pregnancy occurring.

b. Model 2

Assessing the 21-day risk periods as described in model 1, mean probability of insemination occurring in a given risk period was 0.364. Mean probability of insemination occurring in a given risk period for a cow with a Currently Negative MAP antibody ELISA status was 0.405; a cow with Uncertain status had a mean probability of insemination in a given risk period of 0.351, with 0.325 and 0.178 mean probabilities of insemination in a given risk period for Provisionally Positive and Positive statuses respectively.

The same explanatory variables were included in model 2 as in model 1, except for the addition of year of risk period. Parameter and variance estimates for model 2 are shown in Table 8; herd level variance was estimated as 0.38 (0.035 standard error) and cow level variance estimated as 0.65 (0.007 standard error).

Associations between current and previous lactation Provisionally Positive and Positive MAP antibody ELISA status and probability of insemination occurring in a given risk period were again significantly negative but of a greater magnitude than those described in model 1. Associations between transition from Currently Negative to any non-negative MAP antibody ELISA status and probability of insemination occurring were also of a similar direction to those described in model 1, with Positive MAP test status transition in the next 30 to 90 days after the end of a risk period demonstrating a smaller negative association with probability of insemination occurring and Positive MAP test status transition in the next 90 to 180 days demonstrating a small positive association with probability of insemination occurring, compared to model 1. In contrast to model 1, in model 2 transition to a non-negative status at >365 days after the end of a risk period had no significant association with probability of insemination occurring.

c. Model 3

A total of 136,691 inseminations were assessed in model 3 after excluding inseminations with missing 305-yield data, with an overall conception risk (risk of an insemination leading to a pregnancy) of 37.67%. Parameter and variance estimates for model 3 are shown in Table 8; herd level variance was estimated as 0.31 (0.029 standard error) and cow level variance estimated as 0.48 (0.016 standard error).

None of the current MAP antibody ELISA status categories were significantly different from the reference category (Currently Negative MAP antibody ELISA status), with only the No Status category significantly different from the reference. Similarly to model 1, transition from Currently Negative MAP antibody ELISA status to any non-negative MAP antibody ELISA status in the next 30 to 90 days after an insemination was significantly negatively associated with probability of pregnancy occurring (OR, 0.65; CI, 0.50 - 0.84) and transition from Currently Negative to any non-negative MAP test status in the next 90 to 180 days after an insemination (OR, 1.26; CI, 1.11 - 1.43) was significantly positively associated with probability of pregnancy occurring. Transition from Currently Negative to any non-negative MAP test status in the next 180 to 365 days after an insemination (OR, 1.08; CI, 0.96 - 1.22) demonstrated a positive relationship with probability of pregnancy occurring, although this was not statistically significant.

Table 8: Parameter estimates for logistic regression final* Models 1^a, 2^b and 3^c

Characteristic	Model 1		Model 2		Model 3	
	OR	95% CI	OR	95% CI	OR	95% CI
305-day milk yield (1,000kg)	1.11	1.07, 1.14	1.21	1.17, 1.23	0.91	0.90, 0.92
(305-day milk yield (1,000kg)) squared	0.99	0.99, 0.99	0.99	0.99, 0.99		
Lactation number						
1	Reference		Reference		Reference	
2	0.87	0.83, 0.90	0.90	0.86, 0.93	0.90	0.86, 0.92
3	0.76	0.72, 0.79	0.74	0.71, 0.76	0.84	0.81, 0.88
4	0.60	0.57, 0.63	0.57	0.54, 0.59	0.76	0.73, 0.80
>4	0.42	0.40, 0.44	0.39	0.37, 0.41	0.64	0.62, 0.68
Current MAP test status						
Currently Negative	Reference		Reference		Reference	
Uncertain	0.94	0.87, 1.02	0.98	0.91, 1.04	1.03	0.96, 1.11
Provisionally Positive	0.77	0.70, 0.84	0.70	0.65, 0.76	0.95	0.85, 1.06
Positive	0.64	0.52, 0.79	0.59	0.49, 0.70	1.12	0.96, 1.30
No status	0.97	0.94, 0.99	0.90	0.90, 0.93	0.92	0.89, 0.97
Previous lactation MAP test status						
Currently Negative	Reference		Reference			
Uncertain	1.00	0.92, 1.08	0.95	0.89, 1.01		
Provisionally Positive	0.86	0.76, 0.95	0.78	0.70, 0.86		
Positive	0.66	0.53, 0.81	0.52	0.43, 0.62		
No status	1.00	0.96, 1.04	0.99	0.96, 1.02		
Currently Negative to non-negative ^d MAP test status within 30 to 90 days after end of risk period (Model 1 and 2) or insemination (Model 3)	0.64	0.53, 0.79	0.78	0.66, 0.92	0.65	0.50, 0.84
Currently Negative to non-negative MAP test status within 90 to 180 days after end of risk period (Model 1 and 2) or insemination (Model 3)	1.46	1.26, 1.68	1.34	1.16, 1.52	1.26	1.11, 1.43
Currently Negative to non-negative MAP test status within next 180 to 365 days after end of risk period (Model 1 and 2) or insemination (Model 3)	1.72	1.36, 2.12	1.70	1.40, 2.08	1.08	0.96, 1.22

Currently Negative to non-negative MAP test status in >365 days after end of risk period (Model 1 and 2) or insemination (Model 3)	2.14	1.27, 3.53				
DIM						
21-41	Reference		Reference			
42-62	8.94	8.14, 9.49	11.02	11.02, 11.02		
63-83	14.44	13.60, 15.49	18.17	18.17, 20.09		
84-104	18.17	16.95, 19.30	24.53	22.20, 24.53		
105-125	16.95	15.80, 18.17	20.09	20.09, 22.20		
126-146	14.87	13.87, 15.96	18.17	16.44, 18.17		
147-167	13.74	12.81, 14.88	14.88	14.88, 16.44		
168-188	11.82	11.02, 12.81	12.18	12.18, 13.46		
189-209	11.25	2.34, 2.51	11.02	11.02, 12.18		
210-230	9.68	10.38, 10.59	9.97	9.02, 9.97		
231-251	9.12	8.25, 9.97	8.17	7.39, 9.03		
252-272	8.00	7.17, 8.85	6.69	6.05, 6.69		
273-293	5.64	5.00, 6.36	4.95	4.48, 5.47		
294-314	4.57	4.01, 5.26	3.32	3.00, 3.67		
315-335	3.35	2.89, 3.94	2.46	2.23, 2.72		
In DIM					6.69	4.48, 11.02
(In DIM) squared					0.83	0.79, 0.87
ISI						
None					Reference	
0-19 days					0.72	0.68, 0.76
19-26 days					1.21	1.17, 1.26
27-37 days					0.97	0.91, 1.03
38-52 days					0.99	0.94, 1.04
>53 days					1.09	1.03, 1.15
Risk period months 2-10	0.74	0.73, 0.76				
Risk period month 1			1.38	1.34, 1.42		
Risk period month 5			1.54	1.49, 1.58		
Risk period month 11 and 12			1.60	1.57, 1.63		
Calving year 2017					0.92	0.90, 0.95

Calving months 3,6, and 7	1.02	1.00, 1.05		
Insemination year 2017 and 2018			1.06	1.03, 1.09
Insemination months 3,7,8,9 and 10			0.95	0.92, 0.97

OR, Odds Ratio; CI, Credible Interval; MAP, *Mycobacterium* subspecies *avium* *paratuberculosis*; DIM, Days-In-Milk; ISI, Interservice Interval

*Final models; all parameters were retained in the model if the 95% confidence interval for their coefficients did not cover zero and if rejected, were reintroduced into the model after initial variable selection and retained if they satisfied the criteria described above. Where one or more categories of a categorical variable were significant, all categories for that variable were retained.

^aModel outcome; cow becoming pregnant in 21-day risk period. Herd level variance was estimated as 0.26 (0.025 standard error) and cow level variance estimated as 0.39 (0.016 standard error).

^bModel outcome; cow receiving insemination in 21-day risk period. Herd level variance was estimated as 0.38 (0.035 standard error) and cow level variance estimated as 0.65 (0.007 standard error).

^cModel outcome; cow becoming pregnant for given insemination. Herd level variance was estimated as 0.31 (0.029 standard error) and cow level variance estimated as 0.48 (0.016 standard error).

^dUncertain/Provisionally Positive/Positive MAP test status.

Results from all models are visually summarised in Figure 8 using posterior predictions for predicted relative risk, for risk of insemination occurring in a 21-day risk period, conception occurring for a given insemination and pregnancy occurring in a 21-day risk period. Differences in temporal association between MAP antibody ELISA test status and risk of 21-day insemination, conception and 21-day pregnancy are clearly visible in this figure.

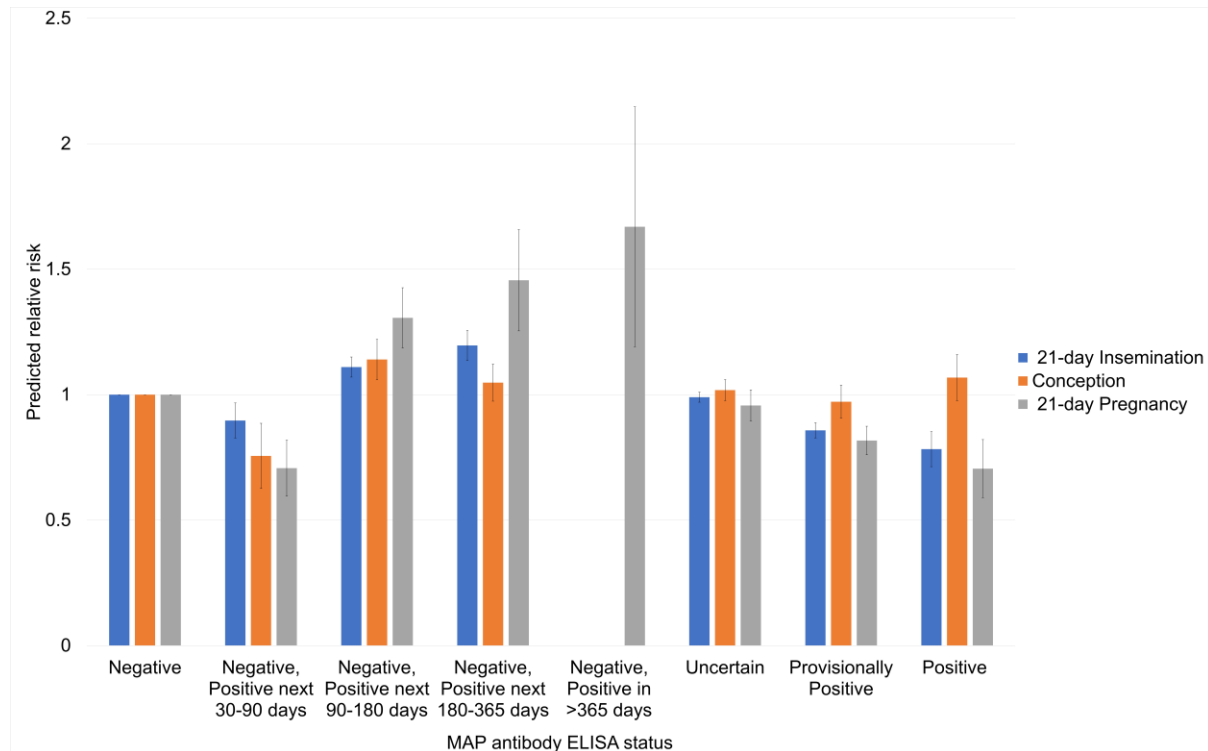


Figure 8: Association between predicted relative risk of insemination occurring in 21-day risk period, conception occurring for given insemination and pregnancy occurring in 21-day risk period and *Mycobacterium subspecies avium paratuberculosis* (MAP) antibody enzyme-linked immunosorbent assay (ELISA) status. MAP antibody ELISA status definitions; negative, currently negative current status; uncertain/provisionally positive/positive, current status; negative, positive next 30-90 days/90-180 days/180-365 days/>365 days, future non-negative MAP antibody ELISA positive status in 30 to 90 days/90 to 180 days/180 to 365 days/>365 days after end of risk period (model 1 and 2) or insemination (model 3).

3.2 Model checking

All the observed proportion of cases in which a pregnancy or insemination occurred lay within coverage of the 95% credible interval of model predictions across all subgroups for all models, indicating acceptable model fit. Comparison of Model 3 predictions for conception risk with observed mean conception risk for a given insemination across different subgroups is illustrated in Figure 9.

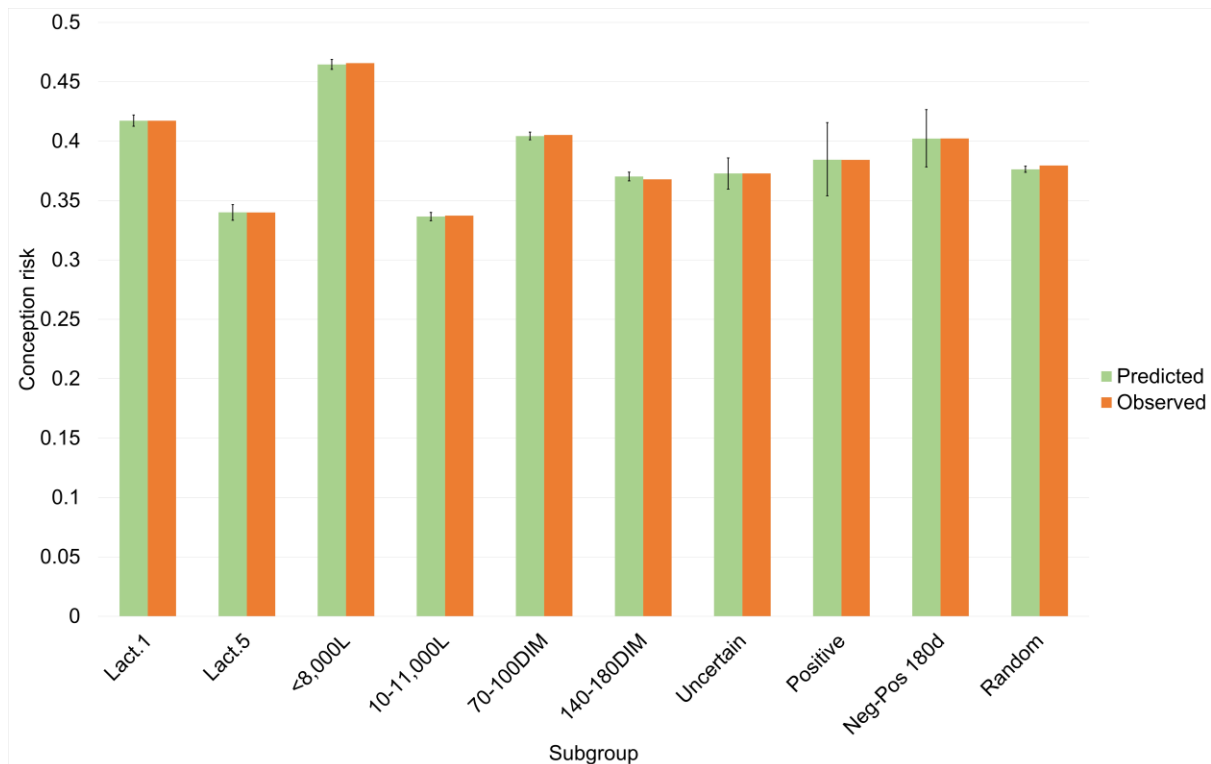


Figure 9: Conception Risk; Predicted versus observed values by subgroup. Orange bars denote mean observed conception risk, green bars denote median predicted conception risk, error bars denote 95% credible intervals of predicted conception risk. Subgroup definitions; lact 1, all inseminations occurring for lactation 1 cows; lact 5, all inseminations occurring for lactation 5 cows; <8,000L, all inseminations occurring for cows with 305 day-yield of less than 8,000L; 10-11,000L, all inseminations occurring for cows with 305 day-yield of between 10,000L and 11,000L; 70-100 DIM (days-in-milk), all inseminations occurring between 70 and 100 DIM; 140-180 DIM, all inseminations occurring between 140 and 180 DIM; uncertain, all inseminations occurring for cows with current uncertain MAP antibody ELISA status; positive, all inseminations occurring for cows with a current positive MAP antibody ELISA status; neg-pos 180 days, all inseminations occurring for cows that transitioned from negative to non-negative *Mycobacterium* subspecies *avium* *paratuberculosis* (MAP) antibody enzyme-linked immunosorbent assay (ELISA) status within next 90 to 180 days; random, a randomly selected subgroup of 50,000 inseminations.

Chapter 4: Discussion

There are significant challenges in accurate diagnosis of paratuberculosis and its consequent association with fertility performance; lack of correct identification of infected animals in a disease with a long latent period, coupled with difficulty temporally associating fertility performance with infection status, especially when measuring interval-based outcomes has resulted in equivocal evidence on MAP test status and fertility in the existing literature.

Using temporal analysis of the association between fertility performance and MAP milk antibody ELISA test status, this novel study has helped contextualise previous contrasting findings reported in literature, whilst also revealing additional temporal associations between insemination and conception risk and occurrence of a MAP antibody ELISA positive result.

4.1 Associations between MAP status and risk of conception for a given insemination

Model 3 was constructed to evaluate risk of conception for a given insemination associated with different MAP statuses; none of the current non-negative MAP antibody ELISA status categories were significantly different from the reference category (Currently Negative MAP antibody ELISA status), however transition from Currently Negative to any non-negative status in the next 90 days and in more than 90 days was significantly negatively and positively associated with probability of conception, respectively.

No previous work has accurately measured association between fertility outcomes and time to a future positive MAP status, however this investigation had been recommended in other literature following positive association between positive MAP status and fertility (Marcé *et al.*, 2009). Both trends found in the current study suggest that conception risk for a given insemination is temporally related to occurrence of a positive MAP antibody ELISA result; inseminations recorded closest to a positive MAP antibody ELISA result were less likely to lead to a pregnancy. This is supported by recent UK work which reported that cows with a recent MAP antibody ELISA positive result were more likely to return to service at 56 days and require more inseminations per conception than cows with a less recent positive MAP antibody ELISA result (Pritchard *et al.*, 2017). Previous work also highlighted an increased calving interval during the period of a positive MAP antibody ELISA result (Sibley, Orpin and Pearse, 2012), although this metric measures occurrence of insemination as well as conception.

However, also in the current study, inseminations associated with a less recent positive MAP antibody ELISA result (Uncertain or Positive current status) or where transition to any future non-negative status occurred more than 90 days after the insemination, were more likely to lead to a pregnancy. This broadly supports previous work documenting improved fertility in cows with a current Positive MAP antibody ELISA status, although recency of MAP antibody ELISA positive result was not reported in these studies (Lombard *et al.*, 2005; Gonda *et al.*, 2007; Marcé *et al.*, 2009; Smith *et al.*, 2010). The MAP antibody ELISA test was the only diagnostic test used to define MAP status in this study with no data collected on clinical signs; other work using faecal culture in addition similarly reported positive association with fertility in MAP antibody ELISA positive and low shedding faecal culture positive cows

compared to antibody ELISA negative cattle, however poorer fertility was demonstrated in high shedding faecal culture positive cows (Smith *et al.*, 2010) and reducing fertility noted in higher parity ELISA positive animals (Marcé *et al.*, 2009) compared to antibody ELISA negative cattle. It could therefore be hypothesised that inseminations associated with a less recent positive MAP antibody ELISA result or a future non-negative MAP status more than 90 days away had an increased risk of conception as these animals may be younger and not yet in a more advanced state of disease, with reduced fertility associated with disease progression.

It is physiologically unclear why these animals have a higher probability of conception than Currently Negative animals; a T_H2 dominated immune response has been implicated in MAP antibody ELISA positive cows and also in the maintenance of a successful pregnancy (Gonda *et al.*, 2007), with this response widely documented in human and mice as pivotal in both establishment and maintenance of pregnancy (Fair, 2015). Both T_H1 and T_H2 immune factors are expressed during early pregnancy in cattle (Fair, 2015), suggesting further work is warranted in this area to define a biological mechanism for this phenomena seen and if this is present in MAP infected cows before first turning ELISA positive. It should also be considered that this association may be reversed; pregnant cattle with a gestation of more than 90 days may be more susceptible to developing a non-negative MAP antibody ELISA test status compared to those with a shorter gestation, although this has been unconfirmed in literature, with a significant increase in seroprevalence only detected in the third trimester in one study (Selim *et al.*, 2021).

Pathogenesis for reduced conception risk associated only with the occurrence of a MAP antibody ELISA positive result is also currently unclear; negative energy balance secondary to granulomatous small intestinal inflammation and consequent reduction in nutrient absorption efficiency (Sweeney, 2011), has long been hypothesised as contributory to reduced conception risk demonstrated in animals with a positive MAP antibody ELISA status (Johnson-Ifeorlundu *et al.*, 2000; Pritchard *et al.*, 2017). Furthermore, a recent study reported significant association between MAP seroconversion and early pregnancy loss, although the sample size for this was small (10 out of 33 cows) and no mechanism for this was established (Garcia-Ispuerto and López-Gatius, 2016). Clearly, further work is required to verify if negative energy balance is present specifically only surrounding a MAP antibody ELISA positive result, during earlier states of disease.

4.2 Associations between MAP status and risk of insemination per 21-day risk period

In model 2, both past and current Provisionally Positive and Positive MAP antibody ELISA statuses were significantly negatively associated with probability of insemination in a 21-day period. This is consistent with findings in previous work (Raizman *et al.*, 2007). Other work has demonstrated that cows with Positive MAP antibody ELISA status were at an increased risk of removal once producers were made aware of test results (Lombard *et al.*, 2005); due to the retrospective nature of the current study it is likely that herd managers had access to test results and suggests that cows with more recent or multiple positive MAP antibody ELISA results may have been declared not to be bred by herd managers. However, transition to any non-negative future MAP status in the next 90 days was also significantly negatively

associated with probability of insemination in a 21-day risk period, before producers would have been aware of a positive MAP antibody ELISA result. The hypothesis for the significant reduction in insemination risk within this close proximity of a positive ELISA result is similar to that for conception risk; negative energy balance associated with MAP infection may be implicated in reduced oestrus expression or increased post-partum anoestrus (Johnson-Ifeorlundu *et al.*, 2000; Butler, 2003; Butler, Pelton and Butler, 2006) although this is currently unconfirmed. Conversely and like the association identified in model 3, transition to any non-negative future MAP status in more than 90 days was significantly positively associated with probability of insemination in a 21-day risk period. A physiological mechanism associating early-stage MAP infection and increasing insemination risk is not hypothesised as for conception risk and warrants further investigation; a T_H2 dominated immune response has been reported as upregulated during the luteal phase of the bovine oestrous cycle although also implicated in success of CL regression in the absence of pregnancy (Fair, 2015), therefore making a T_H2 dominated immune response likely more relevant in success and maintenance of CL function and conception than oestrous expression or cyclicity.

4.3 Associations between MAP status and risk of pregnancy per 21-day risk period

Results from model 1 represent a combination of the associations from models 2 and 3; both past and current Provisionally Positive and Positive MAP antibody ELISA statuses were significantly negatively associated with probability of pregnancy in a 21-day risk period which was predominantly driven by significant reduction in insemination risk for these statuses (model 2) and likely producer bias. Transition to non-negative future MAP status in the next 90 and more than 90 days was significantly associated with a decrease and increase in probability of pregnancy in a 21-day risk period, respectively; this was strongly affected by the size and direction of both insemination and conception risk for these statuses in models 2 and 3 and demonstrates the potential impact proximity of a MAP antibody ELISA positive result may have on overall fertility.

The projected costs correlated with these associations are outlined in Table 9; predicted median 21-day pregnancy rate per MAP status is derived from median posterior prediction for probability of pregnancy per 21-day risk period, whilst values of all other explanatory variables were set at the population mean, with a UK cost estimate of £16 per every % gain in 21-day pregnancy rate per cow per year used, as reported in literature (Russell, 2014). It must be noted that even for current MAP antibody ELISA positive cattle, although below the commonly quoted target of 20-25%, the 21-day pregnancy rate is above that of the recently calculated national median (16%) (Hanks and Kossaibati, 2022). As mentioned in Section 4.4, use of non-probability sampling may have led to selection of herds with better management of fertility, restricting generalisation of associations to similar herd types. However, clearly economic impact of reduced 21-day pregnancy rate may be significant in higher prevalence herds, with greater impacts of disease possible in herds with poorer management.

Table 9: Estimated change in revenue per cow per year (£) based upon predicted median 21-day pregnancy rate, across varying MAP antibody ELISA status

MAP antibody ELISA status	Predicated median 21-day pregnancy rate (%)	Estimated change in revenue/cow/year (£)
<i>Current lactation status</i>		<i>- vs Currently Negative cattle</i>
Currently Negative	24.62	Reference
Uncertain	23.56	-16.96
Provisionally Positive	20.14	-71.68
Positive	17.36	-116.16

4.4 Study limitations

Considering the reduced sensitivity of the antibody ELISA assay used in this study (Chapter 2.1.c), it is therefore more likely that infected cattle may have falsely tested antibody ELISA negative than non-infected cattle testing positive, although use of different MAP antibody ELISA test categories derived from multiple recent antibody ELISA results helped reduce the risk of mis-classification bias (Table 2).

Use of non-probability sampling techniques represent a possible limitation in this study; data was only selected for analysis from herds with complete and more accurate records. The nature of such herds in the UK suggests that this may have created a selection bias towards herds that are larger, with more intensive production and prudent management. This makes generalisation of associations between MAP antibody ELISA status and fertility more likely among similar types of herd, although it is possible that biological differences at cow level may be smaller. It should also be noted that generalisation of these associations across herds using different milk antibody ELISA assays is reduced, due to potential variation in ELISA assay steps and consequent assay characteristics. A degree of sampling and measurement bias may impact the external validity of study findings when applying these to the national population of dairy herds in the UK. However as later discussed, acceptable model fit and lack of overparameterization in all models increases transferability of study outcomes to the wider population. This does enable herds to generally consider the temporal associations of positive MAP antibody ELISA result with insemination and conception risk on a more practical basis, which is discussed in Chapter 4.5.

As this was a retrospective observational study based upon secondary data, this type of work can only establish associations, with further work required to demonstrate causality. Furthermore, lack of prospective design resulted in a lack of control of type of outcomes measured, accuracy of data recording and the need for integration of data from multiple sources, all with potential to impact the quality of data analysed. Novel data quality assessment criteria were therefore developed and applied in this study to help moderate the impact of these aspects on accuracy and completeness of data. A total of 203,945 lactations from 44,478 cattle in 15 herds were removed from analysis following data quality screening and removal of lactations with a calving event prior to 2010 to maximise presence of MAP test results for analysis; basic statistics describing these herds are detailed in Table 3, with particular improvement clear in fertility-based metrics following data quality screening. Despite removal of this data, a large number of each MAP antibody ELISA test

category remained for analysis, with 183,175 currently negative statuses, 8,785 uncertain statuses, 4008 provisionally positive statuses and 2770 positive statuses (Table 4).

The aim of this study was to assess the association between MAP antibody ELISA status and fertility performance across a large sample of commercial UK dairy herds, whilst retaining the ability to evaluate associations temporally within each lactation. No sample size calculations were performed prior to analysis, however a variety of statistically significant associations were established between past, current and future MAP statuses in most models, demonstrating adequacy of the sample size used. Nonetheless, no significant associations were established between any non-negative current MAP status and risk of conception for a given insemination, however these followed similar trends as those significant associations identified between risk of conception and future non-negative MAP status, suggesting that a larger sample size may have revealed statistical significance.

After initial posterior predictive assessment and categorical reclassification of the DIM variable in Models 1 and 2, regeneration of full posterior predictions and comparison to the observed mean indicated acceptable model fit across all samples tested, in all models. However, use of within-and-out-of-sample and 10-fold cross validation demonstrating AUROC for each model of less than 5% difference across sampling methods, indicated lack of overparameterization.

4.5 Farm management implications and future studies

Potential practical applications of study outcomes on farm are not extensive; it is important however that producers are aware that MAP infected animals which are currently still MAP antibody ELISA test negative may be more likely to be inseminated and then conceive than truly uninfected animals. This is significant when considering the potential for retaining and breeding MAP infected animals, especially when many milk supplier contracts require a minimal paratuberculosis prevalence. Producers may use perceived fertility performance to help make herd breeding decisions, with better performing animals more likely to be bred to produce replacement heifers for the future herd. If future MAP positive animals are chosen for this, this would increase the chance of recycling and retaining MAP infection within the herd, however, this is more likely in herd with a higher prevalence of MAP infection. Management of this aspect is likely to be best achieved by repeated individual cow and breeding heifer antibody ELISA sampling and using dam antibody ELISA results for breeding heifer selection, to help guide breeding and management decisions and maximise detection of infected animals.

However, in herds without a high prevalence of MAP infection cattle may be present at a range of infection stages. This may make it unlikely herds experience significant negative impacts on fertility performance, with potential for positive and negative associations to prove equivocal.

It is important to note that this study has demonstrated associations between positive MAP antibody ELISA status and fertility performance, with future studies required to establish causality and potential biological mechanisms. Reduced insemination and conception risk around the time of a positive MAP antibody ELISA result has been hypothesised to be

associated with presence of negative energy balance during this period. Future work tracking status of negative energy balance alongside that of MAP infection and fertility performance in individual cows, could help establish this at a more granular level. Similarly, experimental infection models could be utilised to investigate potential immunological or other mechanisms present positively impacting chance of insemination and conception within the infected individual cow, prior to turning antibody ELISA positive.

However, although this work would advance understanding of disease pathogenesis, this may not change practical application of fertility management on farm. Further assessment of antibody ELISA data to establish any associations between antibody ELISA titre and consequent fertility performance may help producers to manage cattle at increased risk of potential reduced fertility performance and aid culling decisions.

4.6 Conclusions

This study has helped provide a clearer picture of the associations between MAP antibody ELISA status and UK dairy cow fertility, amidst a large amount of variability in existing literature. Measurement of time to a positive MAP antibody ELISA result has enabled temporal association with fertility outcomes and helped contextualise findings from previous studies which otherwise provided conflicting evidence.

This study demonstrates a variable but largely positive relationship between non-negative MAP antibody ELISA status and cow fertility outcomes prior to a positive MAP antibody ELISA result; negative fertility associations appear leading up to and around the time of a positive MAP antibody ELISA result, with only a long-term negative relationship between insemination risk and non-negative MAP status thereafter. This demonstrates the potential impact of subclinical MAP infection on dairy cow fertility and consequently herd economic return, before producers are aware of production losses or clinical signs. This highlights the importance of assessing herd prevalence by regular antibody ELISA testing to help control disease and maximise herd sustainability.

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Appendices

Appendix 1: Requirements for a Standard Residency Programme in an approved institution, for admission to ECBHM examinations

Requirements	Detail	Fulfilment
At least 3 years of full-time training under ECBHM Diplomate supervision	66% in clinical activity: - 33% at individual animal level - 33% at herd level 33% partaking in research and scholarly activity, preparing scientific manuscripts, graduate degree studies and externship rotations.	Total individual animal cases: 554 Total herd health cases: 346 Externship at University College Dublin: 27/02/23-10/03/23 See below for research/scholarly activity and manuscripts
Veterinary anatomic and clinical pathology	Minimum of 1 week actively participating in a clinical pathology laboratory and in 30 bovine necropsies	Pride clinical pathology laboratory: 03/05/21-07/05/21 Necropsy cases: 34
Conferences	Attend internal sessions (<i>i.e.</i> rounds, journal club) Participate in 2 ECBHM Residents workshops Attend 3 national/international conferences Present at 1 national/international conference	Lectures/seminars attended: 226 ECBHM workshops attended: 2 Conferences attended: 8 Presentations at conferences: 2 - BCVA October 2021: 'Fertility data quality assessment' Reynolds J., Hudson C. - BCVA October 2022: 'Associations between Johne's Disease and fertility in UK dairy herds' Reynolds J., Bradley A., Sherwin V., Remnant J., Hudson C.
Seminars	Present minimum of 6 seminars of 45-minute duration	Lectures/seminars given: 31
Teaching	Participate in clinical education of undergraduate and graduate veterinary students	Participation in teaching Year 1 to 5 undergraduate veterinary students and mentoring graduate veterinary surgeons in clinical practice Awarded University of Nottingham Postgraduate Teaching Award 2022 for recognition of

		innovative teaching and support of learning.
Research	Completion of investigative project contributing to advancement of bovine health management, in >12 months	Completion of Masters in Veterinary Medicine 'Associations between Johne's Disease and fertility in UK dairy herds'
Scholarly output	Two publications in field of bovine health management in peer-reviewed scientific journals: - One original research paper - One case report/original research paper/review paper Five case reports (minimum 1 individual animal)	Publications: - Reynolds J., Bradley A., Sherwin V., Remnant J., Hudson C. (2023). "Associations between Johne's Disease and fertility in UK dairy herds". <i>The Veterinary Journal</i>; DOI: 10.1016/j.tvjl.2023.106015 - Reynolds J., Hudson C. (2023). "Herd health: approach to assessing fertility data quality". <i>In Practice</i>; 45(4); DOI:10.1002/inpr.311 - Reynolds J., Brennan M. (2021). "Is thoracic ultrasound more efficient than the Wisconsin calf scoring system for the detection of pneumonia in calves?". <i>Veterinary Record</i>; 189(2); DOI: 10.1002/vetr.752 Five case reports: passed
Progress evaluation	Bi-annual formal supervisor meetings Annual progress report: case logs and case report(s)	Completed

Appendix 2: Requirements for passing ECBHM certifying examination

Requirements	Detail	Fulfilment
Credentials for admission to examination	Fulfilment of detail in Appendix 1 Letter of reference Curriculum vitae	Passed
Section A: basic knowledge and reflection	Paper 1: single best answer questions on recent publications on bovine health management Paper 2: single best answer questions on basic clinical knowledge Paper 3: clinical exhibit short answer questions	Passed (>60%)
Section B: problem solving and communication	Paper 1: two problem-based herd health questions Oral examination: - Presentation of research/discussion - Discussion on 'hot topic' research	Passed (>60%)

Appendix 3: European Board of Veterinary Specialisation Certificate



Appendix 4: RCVS Specialist Certificate



*Royal College
of Veterinary
Surgeons*

This is to certify that

Jessica Amy Reynolds

BVM BVS DipECBHM MRCVS

is recognised as a

Specialist in Cattle Health & Production

and has fulfilled the required criteria for the period

01 September 2023 - 31 August 2028

A handwritten signature in black ink, appearing to read 'E. Egan'.

Registrar

