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RUNNING HEAD: BOTTLENECKS IN JOHNE'S DISEASE CONTROL

Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection

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Summary

In the last decades, many regional and country-wide control programs for Johne's disease (JD) were developed due to associated economic losses, or because of a possible association with Crohn's disease. These control programs were often not successful, partly because management protocols were not followed, including the introduction of infected replacement cattle, because tests to identify infected animals were unreliable, and uptake by farmers was not high enough because of a perceived low return on investment. In the absence of a cure or effective commercial vaccines, control of JD is currently primarily based on herd management strategies to avoid infection of cattle and restrict within farm and farm-to-farm transmission. Although JD control programs have been implemented in most developed countries, lessons learned from JD prevention and control programs are underreported. Also, JD control programs are typically evaluated in a limited number of herds and the duration of the study is less than 5 y, making it difficult to adequately assess the efficacy of control programs. In this manuscript, we identify the most important gaps in knowledge hampering JD prevention and control programs, including vaccination and diagnostics. Secondly, we discuss directions that research should take to address those knowledge gaps.

Keywords: Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis*, control, prevention

Introduction

Johne's disease (JD) is an infectious chronic inflammatory disorder of the intestine in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is a major health problem, resulting in intermittent diarrhea, loss of body condition, and lower productivity (e.g. Tiwari et al., 2006). In the terminal phase, which most animals will not reach due to premature culling, animals die in very poor body condition. Infected ruminants shed MAP in manure and milk in increasing quantities as the disease progresses (Whitlock and Buergelt, 1996). The disease is widespread in domestic and wild ruminant populations in almost all countries in the world and causes great economic losses, not only because of lower productivity, but also as a result of loss of future income due to premature culling (McKenna et al., 2006b; Garcia and Shalloo, 2015). The herd-level prevalence of MAP infection is likely >50% in most countries with a substantial dairy industry (Barkema et al., 2010). In the absence of control measures, JD typically spreads, as farms often purchase cattle, frequently from herds with unknown JD status. In a recent review, Garcia and Shalloo (2015), reported substantial losses due to MAP infection, which escalate as the within-herd MAP prevalence and incidence of clinical JD cases increase. In Canada, the economic damage caused by JD was estimated at $50 CAN per cow per year in MAP-infected herds, resulting in an average loss per infected farm of nearly $3,000 CAN annually (Chi et al., 2002; Tiwari et al., 2008). Raizman et al. (2009) estimated the income over feed cost losses at $366 per MAP-shedding cow per lactation, whereas Bhattarai et al. (2014) estimated a loss of $1,644 US

per 100 cows in a herd with a true prevalence of 7%. The cost of the disease to the US cattle industry was estimated at $250M US per year (Ott et al., 1999).

Meta-analyses have demonstrated that the association of MAP with Crohn's disease in humans is specific and cannot be denied (Feller et al., 2007; Abubakar et al., 2008), although a causal role has not yet been demonstrated (Waddell et al., 2015; Waddell et al., 2016). Furthermore, transmission from cattle to humans has never been proven. However, addressing JD worldwide should be considered a proactive step in ensuring consumer confidence if a link were to be established between JD and Crohn's disease.

The epidemiology of JD is very different among cattle, sheep and goats. These species have very distinct differences when it comes to the course of MAP infection; therefore, inferences from one species cannot be naively applied to other species. Production type (e.g. dairy versus beef) also has a role in the course of MAP infection. This is not only evident in cattle (dairy versus beef), but probably even more evident in sheep and goats (i.e., wool/meat producing sheep in Australia and milk producing sheep/goats in the Mediterranean).

In this manuscript, we identify the most pressing gaps in knowledge hampering JD prevention and control programs (summarized in Table 1). Secondly, we discuss directions that research should take to solve these knowledge gaps.

Control programs

Due to economic losses, and its possible association with Crohn's disease, many control programs for JD have been developed worldwide. Many of these programs

focused on MAP-infected herds and were based on testing and culling test-positive cows, plus management adaptations (Benedictus, 1984; Collins, 1994; Rossiter et al., 1996; Kennedy, 2001). The focus of some other programs was to identify MAP-negative herds with the aim of keeping these herds, and in the case of Australia, Norway, and Sweden an entire region negative, and also having them as a source of MAP-negative replacement animals (e.g. Kalis et al., 2004; Kennedy et al., 2011; Frössling et al., 2013; Whist et al., 2014). Tests included delayed type hypersensitivity (skin) tests, serological tests, direct detection of MAP by microscopy, and using culture or polymerase chain reaction (PCR) to detect MAP in fecal samples. Presently, fecal culture is considered the most sensitive and specific ante-mortem test to identify MAP infection (Kalis et al., 2002; Whitlock et al., 2000). However, as individual fecal culture is expensive and time consuming, most JD control programs use ELISAs to detect potentially infected animals (e.g. Lavers et al., 2014). Another reason for using ELISAs is that the extent of shedding correlates well with the ELISA titers (Dargatz et al., 2001), hence culling ELISA-positives may be an effective and relatively low-cost option for removing high shedders. Currently, fecal culture is often replaced with direct PCR on feces (e.g., Plain et al., 2014; Laurin et al., 2015).

JD control programs have been implemented in most developed countries, with objectives based on the national economic situation of the cattle, sheep and goat industry and the herd-level prevalence of MAP infection (reviewed by Kennedy, 2011; Geraghty et al., 2014). In general, objectives include: 1) prove and protect freedom of disease at the country, regional or farm-level, e.g. in Norway (Whist et al., 2014), Sweden (Frössling et al., 2013), and northern and western Australia (Kennedy, 2011); 2) protect export of milk

or genetics, e.g. Canada (McKenna et al., 2006a); 3) decrease prevalence of MAP infection and limit farm-level economic losses, e.g. Denmark (Nielsen et al., 2007), the UK (Pritchard et al., 2017), Ireland (McAloon et al., 2016) and the USA (Wells et al., 2008); 4) eliminate or reduce MAP load in bulk milk, e.g. the Netherlands (Weber and van Schaik, 2007); and 5) eliminate MAP infection, e.g. Norway in goats (Nagel-Alne et al., 2014).

Some factors that hampered the success of such control programs were lack of compliance with management protocols, use of tests with a sufficiently high sensitivity to identify infected cattle, persistence of MAP in the environment, inadequate test frequency, appearance of unexpected new infections, and purchase of replacement animals causing new introductions (Benedictus, 1984; Collins, 2001). A complementary modeling study showed that consistent application of preventive measures was key to success, although only "test and cull" was not crucial to control JD (Groenendaal et al., 2003). However, "test and cull" can support and hasten elimination of infection from a herd that also has good management practices (Kudahl et al., 2011). Because there is no cure and, except for sheep, there are no vaccines that effectively prevent MAP infection, control of JD is currently based primarily on herd management strategies that reduce the risk of infection in young calves and restrict within-farm and farm-to-farm transmission. Additionally, control programs that include testing of individual animals, in general advise culling of MAP-positive animals.

Although JD control programs have been implemented in most developed countries, the lessons learned from the actual experience with JD prevention and control programs are underreported worldwide. JD control programs are typically evaluated in a

small number of herds (e.g. Collins et al., 2010; Pillars et al., 2011) and many cattle control programs have not included beef cattle. Additionally, particularly in cattle, results of a JD control program can only be judged after at least 5 y (Caldow and Gunn, 2001; Nielsen and Toft, 2011), although most of the current well-designed programs have been implemented that long (Table 1). Except for goats in Norway (Nagel-Alne et al., 2014), no report could be found of a herd in which MAP infection has successfully been eradicated (Table 1). There are many explanations for this failure which are discussed in the following sections.

Finally, some programs were implemented but abandoned due to the outbreak of another disease (e.g. Bovine Spongiform Encephalopathy or Foot-and-Mouth Disease) or loss of funding (e.g. USA and Alberta, Canada), making it difficult to regain trust and re-institute the program.

Prevalence

It is essential that reliable estimates of disease prevalence (animal or herd level) are available since this will determine how to proceed and monitor the results of a control program over time. The goals may vary from eradication in areas of low prevalence, control in areas with high prevalence, or increased surveillance in an area with no prior history of disease. Prevalence estimates obtained by surveys are affected by test accuracy; therefore, comparisons among studies must be adjusted to better estimate the true prevalence.

Infection with MAP and cases of JD have been reported from all continents with ruminant populations. Prevalence in most regions is currently unknown, and prevalence studies have low design uniformity, making comparisons among regions unreliable due to different sampling strategies and case definitions. Additionally, in most studies, the true herd- and animal-level prevalence has not been estimated. Thus, the results of these studies and the reported prevalence estimates of MAP infection can currently not be compared directly (Table 1). Additionally, in most regions, herd and animal-level prevalence are underestimated, as the sensitivity of ELISAs is overestimated, particularly for cattle in 1st lactation heifers (McKenna et al., 2005a,b; Nielsen and Toft, 2008). Almost invariably, in regions where herd-level prevalence estimates were obtained using an ELISA and independently validated using fecal culture or environmental culture, true prevalence estimates (adjusted for test characteristics) are much lower based on testing with ELISA versus culture (e.g. Lavers et al., 2014; Wolf et al., 2014). Although MAP test comparisons have been the subject of many studies, there is a lack of studies comparing test characteristics in populations that reflect the target population when estimating prevalence of MAP infection (Nielsen and Toft, 2008). The sensitivity of MAP tests increases in dairy cattle with increasing days in milk and age but decreases with increased milk yield (Eisenberg et al., 2015b; Kirkeby et al., 2015), whereas sensitivity increases with age in sheep and goats (Lybeck et al., 2011). Therefore, sensitivity estimates need to be adjusted for age in all ruminants, and days in milk and milk yield in dairy cattle when comparing between or within herds and also over time.

Comparing prevalence over time, among regions and countries is often unreliable due to the use of a variety of diagnostic tests, often with unknown or unreliable test

characteristics. To compare herd- and animal-level prevalence estimates of MAP among countries, and to allow for the development of international trade standards, we recommend a supranational standardized study, comparable to a *Neospora caninum* seroprevalence study involving cattle from 4 countries (Bartels et al., 2006) (Table 1).

The Pathogen

Strain typing is useful in helping to clarify epidemiological and virulence questions that cannot otherwise be resolved; however, limited genetic variation among MAP isolates within host species has slowed progress. Several typing techniques have been used (Collins and de Lisle, 1986; Coffin et al., 1992; Whittington et al., 1998; Whittington et al., 2000; Motiwala et al., 2003; Amonsin et al., 2004; Thibault et al., 2007). Whole-genome sequencing (WGS), because of its higher resolution, is the logical evolution of technology to assist epidemiological investigations and control programs. The cost of WGS still has to decrease though for this technique to become the technique of choice in this kind of studies.

Variable number tandem repeat (VNTR) and mycobacterial interspersed repetitive unit (MIRU) typing have been moderately successful for characterizing MAP isolates. Diversity among isolates has been reported based on VNTR typing (Thibault et al., 2008; Stevenson et al., 2009; Fritsch et al., 2012; Sohal et al., 2014), with most isolates being 1 of 2 dominant VNTR types. MIRU-VNTR genotyping does not, however, accurately reflect phylogenetic relationships, and repeat sequences are subject to homoplasy (Ahlstrom et al., 2014; Bryant et al., 2016). Current VNTR typing includes loci that are

too unstable and unreliable to be used for a molecular epidemiological analysis of MAP (Ahlstrom et al., 2015). Short sequence repeat (SSR) typing (Amonsin et al., 2004) further differentiates MAP isolates of the same VNTR type (Stratilo et al., 2006; Thibault et al., 2008) but lacks sufficient discrimination and stability for epidemiological studies (Kasnitz et al., 2013). Nevertheless, in specific cases, VNTR (with or without SSR typing) can still be useful for supporting source tracing investigations and JD control measures (Oakey et al., 2014).

Whole-genome sequencing (WGS) provides unparalleled detail regarding genetic profiles of MAP. Genomic epidemiology of MAP is new (Ahlstrom et al., 2015; 2016) but has already provided important insights into transmission dynamics. Single nucleotide polymorphisms (SNPs) identified through WGS are evolutionarily stable and can be reliably used to identify true evolutionary relationships (Pearson et al., 2009). In a MAP-endemic environment, this level of detail is invaluable in understanding the molecular epidemiology and transmission dynamics. For example, there are at least 8 genetically distinct MAP subtypes in Canadian dairy cattle, with > 80% of isolates belonging to a single dominant subtype (Ahlstrom et al., 2016). However, this information cannot be extrapolated to other countries because dominant strains vary between countries and continents (Whipple et al., 1990; Machackova et al., 2004; Motiwala et al., 2004; Kiehnbaum et al., 2005). Some strains may be more successful in spreading or persisting in different environments or management systems. It is difficult to know whether high MAP prevalence within a specific herd relates to a high MAP infection opportunity (e.g. poor biosecurity) or is due to MAP strains with increased transmission potential on these farms.

Strain typing can be used to track transmission of MAP in a variety of settings and host species. Studies of within-herd spread of MAP infection (Pradhan et al., 2011), between-herd transmission (Oakey et al., 2014), and the role of wildlife in spreading MAP (Fritsch et al., 2012) require molecular tools that can differentiate MAP types. WGS was recently used to identify multiple mixed genotype infections (Davidson et al., 2016); these infections could affect disease dynamics and impact pathogen evolution and genetics and thereby impact the efficacy of therapeutic treatments or vaccines (Table 1).

An understanding of the full genetic diversity of MAP is needed to accurately assess subtype-specific phenotypes and virulence characteristics, and to develop vaccines and effective management practices (Table 1). Investigations into strain-specific differences in MAP virulence and pathogenicity have been mostly limited, however, to major strain types (Janagama et al., 2006; Motiwala et al., 2006; Gollnick et al., 2007; Borrmann et al., 2011), with significant differences between Types I and II isolates reported for growth rates and intracellular survival (Table 1).

Investigations have determined phenotypic differences among MAP subtypes for a variety of traits, including growth rates and invasion efficiencies, immunogenicity, virulence as measured by macrophage invasion efficiencies and kinomic responses (Whittington et al., 2011; Griebel et al., 2014). Future vaccine development and molecular epidemiological studies should also consider the relative frequencies of MAP subtypes with a focus on dominant subtypes. There are differences among MAP strains in the immune response that they stimulate, as well as differences in host tropism, disease phenotypes and ability to evade control by vaccines (Sohal et al., 2010; Colavecchia et al., 2016) (Table 1).

Tests

Purpose of testing

Test results used in control programs should primarily assist decision makers achieve a specific objective. Furthermore, the specific test strategies employed are likely to differ between regions, depending on the logistics of implementation and other practicalities, as well as herd size (Dorshorst et al., 2006). Identification of infected animals before they spread the infection while still maximizing the life-time production of an animal is the goal of a cost-effective test strategy. Purposes of testing primarily include: 1) estimate infection prevalence to determine the best course of action; 2) minimize financial losses as a result of impaired milk production or growth and increased culling rate; 3) estimate the effects of proposed control measures or evaluate additional measures or management changes; 4) eliminate infectious cattle to reduce spread of infection, 5) reduce the risk of MAP contamination of food products for human consumption; and 6) eradicate MAP from a herd (or region). Tests for these purposes can focus on individual animals, herds, or a subset of the herd. Control strategies that focus on the individual animal and target specific management conditions contributing to transmission include: 1) identification of infectious animals actively shedding MAP; and 2) identification of infected animals at risk of shedding MAP (Table 1).

Ideal tests used for control should identify the infectious animal or predict its infectivity. However, since we do not know the infectious dose of MAP, shedding

patterns of infected animals, and factors contributing to disease progression, it is difficult to interpret test results (Table 1). Consequently, we struggle to identify MAP infection cases and non-cases, which is essential when evaluating the accuracy of diagnostic tests. This has resulted in decades of reported test evaluations that are of questionable quality (Nielsen and Toft, 2008).

Existing tests are performing reasonably well in detecting advanced stages of JD, although the specificity of test results may be challenged and the number of bacteria excreted in feces or milk is rarely quantified. Detection of early-stage infection is only of interest for a control program if these animals then become infectious (Marcé et al., 2011). Otherwise, these infections may disappear from a specific population. However, because the sensitivity of diagnostic tests in calves is generally low, young stock are rarely tested for MAP. As a result, no longitudinal studies have been published that followed infected calves or lambs and confirm how many subsequently test positive (Table 1).

Early-stage diagnostics

Early stage diagnostics primarily target pro-inflammatory immune responses. To be useful, however, they should differentiate infected from recovered animals (Dennis et al., 2011) and indicate if or when an animal will become infectious (de Silva et al., 2013). Whereas detection of fecal shedding by direct extraction followed by a MAP-specific PCR can detect if an animal is infectious (Fock-Chow-Tho et al., 2017), it cannot indicate

whether or when it will become infectious. Such a test would likely be useful to achieve eradication in low prevalent populations (Table 1).

Identification and multiplexing of antigens that elicit an early humoral immune response could yield a serological test that overcomes the current delay in antibody-based detection of MAP infection. Analyses of longitudinal serum samples (from experimentally infected calves) were used to detect developing humoral immune responses against MAP proteins (Bannantine et al., 2008), with anti-MAP antibodies detected as early as 70 d after infection. Novel MAP antigens for detection of antibody responses in subclinical cattle were also reported (Facciuolo et al., 2013).

The interferon-gamma (IFN-γ) assay detects cell-mediated immune responses to early-stage MAP infection (Stabel, 2000), but needs improvements and standardization as differences in composition of the purified protein derivative (PPD) antigens can influence specificity (Kalis et al., 2003; Capsel et al., 2016). Single proteins to increase specificity of T-cell responses are being sought (Huygen, 2014; Carlos et al., 2015; Leite et al., 2015; Rana et al., 2015). Blood samples must be tested within 8 h for optimal results to ensure T-cell viability, although IL-12 and anti-IL-10 supplementation (Mikkelsen et al., 2012) or IL-7 and IL-12 supplementation (Plain et al., 2012) can increase the interval to 48-hour interval before testing. Ultimately, an on-farm test would be most effective.

Leukocyte markers associated with MAP infection may also be used as a diagnostic test. For example, increased expression of CD25 and CD45RO on T cells may be an early indication of infection (Stabel and Robbe-Austerman, 2011), but these markers lack specificity. Furthermore, phenotype analysis of leukocytes is again dependent upon the isolation and analysis of viable cells. The lack of useable and rapid

diagnostic tests for specific biomarkers is clearly a bottleneck in identifying animals with MAP infection.

To accommodate the discovery of biomarkers that can predict the onset of the infectious stage, experimental infection models presenting a natural disease outcome (e.g. Begg et al., 2010; Mortier et al., 2013) are essential for test development (Hines et al., 2007; Begg and Whittington, 2008). Ultimately, longitudinal sampling of herds with different prevalence profiles is necessary to validate the outcomes of experimental infections in an unbiased and representative set of samples from infected but not yet infectious animals.

Late-stage diagnostics

Late-stage diagnostic tests detect anti-inflammatory immune responses, e.g. ELISAs detecting IgG1. They are relatively well characterized and easy to automate, but sensitivity is in general still low compared to viral infections, such as bovine leukosis (Nielsen and Toft, 2008). Ideally, these should be reliable point-of-care tests using blood or milk from individual cows (Lavers et al., 2014) (Table 1). Better characterization of disease progression in relation to the induction of IgG1 antibody responses is desired.

There might be value in detection of immunoglobulin isotypes other than IgG (e.g. IgM and IgA) (Table 1). Although IgM is the first isotype to appear in response to an infection, this response has limited applicability as it transient (Abbas and Riemann, 1988). Isotype switching results in local mucosal production of IgA and anti-MAP IgA responses have been detected in fecal samples from infected sheep (Begg et al., 2015).

The challenge remains the identification of individual MAP proteins that detect antibodies specific to MAP and not environmental *Mycobacterium* spp.

Calves, kids and lambs were reported to be ELISA-positive for MAP 3 to 4 mo after infection (Storset et al., 2001; Kurade et al., 2004; Mortier et al., 2015a). However, in very young animals, such ELISA antibodies may be an indication of passive immunity (transfer of colostrum-derived maternal antibodies), as maternal antibodies reacting with MAP were detected in serum collected from calves up to 121 d of age (Schillinger et al., 2013).

The titer of an ELISA is predictive for the probability of MAP shedding (Weber and Van Schaik, 2007). This association renders the utility of an ELISA as highly applicable to JD control in dairy herds when the aim is to reduce transmission by selective removal of shedders. In that instance, an ELISA may replace fecal culture or PCR but it is then important to shorten the testing interval (Lu et al., 2008).

Herd-level diagnostics

Environmental sampling is a quick sampling method to determine the MAP infection status of a herd (Wolf et al., 2017). However, this sampling method is only sufficiently sensitive in relatively intensive livestock operations such as housed dairy cattle. In extensive farming, such as sheep and cow-calf herds in many countries, environmental sampling currently does not have sufficient sensitivity (Whittington et al., 2003). For example, culture followed by PCR on 6 environmental samples detected 70% of MAP-infected dairy herds (Wolf et al., 2015a). Samples collected from alleyways of

lactating cow pens and manure lagoons were most sensitive (Wolf et al., 2015a). On farms with only manure piles (or if an outdoor lagoon is inaccessible due to weather), additional samples should be collected from indoor manure pits or alleyways. In a recent German study in cow-calf herds, environmental samples collected in high cow traffic areas at the end of the winter had a sensitivity of 64% (Klawonn et al., 2016), which is similar to housed dairy herds (Wolf et al., 2017). There is certainly room for improvement of environmental sampling; modifications in the number of collected samples per set and in sampling intervals may result in more accurate diagnostic protocols (Table 1). Further refinements of environmental sampling, including determining impact of season, are needed (Wolf et al., 2015a). Culture of pooled fecal samples, most often consisting of pools of 5 or 10 for cattle, or 10 to 50 for sheep fecal samples, has been extensively evaluated and proven to be relatively sensitive (Whittington et al., 2000a; Sergeant et al., 2002; Van Schaik et al., 2007; Verdugo et al., 2014b).

Processing pooled environmental samples with direct PCR instead of culture reduces processing time and costs (Boelske and Herthnek, 2010). An additional advantage of PCR compared to culture is, that PCR is not dependent on viable MAP bacteria in the sample. That might result in a higher sensitivity with PCR, especially if samples are collected in the winter, or at locations where manure accumulates over extended times. Test characteristics for PCR (typically using IS*900* or F57 as the target) relative to culture have been reported, with overall good results (e.g. Cook and Britt, 2007; Clark et al., 2008; Fock-Chow-Tho et al., 2017) for both pooled and environmental

samples (e.g. Kawaji et al., 2007; Mita et al., 2016). Results are, however, highly depend on the extraction method and the primer(s) used (Mita et al., 2016) (Table 1).

Testing of bulk tank milk or a pool (whole herd set of Dairy Herd Improvement milk samples) using a commercial ELISA is an inexpensive method of herd screening for JD in dairy cattle (Kelton et al., 2014). To determine a herd's MAP infection status, bulk milk samples can either be analyzed for the presence of MAP-specific antibodies using ELISA, or for the presence of MAP bacteria using direct PCR. Although repeated bulk tank test results are consistent over short intervals and correlated with herd prevalence (Nielsen and Toft, 2014), results can be influenced by herd size and within-herd prevalence of ELISA-positive individuals. This testing identifies herds with moderate (>5% within-herd milk ELISA-positive) to high (>8% within-herd milk ELISA-positive) prevalence (Serraino et al., 2014). This test is not sensitive enough to detect low prevalence herds (Jayarao et al., 2004; Van Weering et al., 2007; Khol et al., 2013) or to monitor subtle changes in within-herd prevalence over time.

As is the case with all JD testing strategies, given the low sensitivity of the tests, the proportion of infected herds is underestimated and negative test results can give dairy producers a false sense of security based on the belief that a negative test means their herd is uninfected. However, with increasing frequency statistical tools such as Bayesian statistics are used to correct for less than ideal test characteristics that are predominantly the result of the biology of MAP infection (e.g. Verdugo et al., 2014; McAloon et al., 2016).

Recent developments and future directions

Novel findings and approaches in diagnostic test development and evaluations are rare, and so far no major breakthroughs have been provided. Mostly, incremental improvements to existing tests and test strategies have been identified, e.g. repeated testing in a Danish JD control program (Nielsen, 2008). Once prevalence is reduced, cost-effective monitoring of the MAP status of a population can be difficult. Development of cost-effective strategies for surveillance and certification are still required while retaining a focus on the fit-for-purpose criterion (OIE, 2012).

Quantitative PCR (qPCR) for assessing the number of MAP bacteria in feces has great promise in comparison to bacterial culture (Christopher-Hennings et al., 2003; Khare et al., 2004; Kralik et al., 2012; Laurin et al., 2015) (Table 1). This technique has been validated on spiked fecal samples and proficiency panels, including fecal samples from naturally infected animals (e.g. Plain et al., 2014). This approach is strongly dependent on efficient DNA extraction, which has been studied (summarized in Bölske and Herthnek, 2010). However, quantification by qPCR is still only a relative method because a standard curve of spiked samples needs to be used to determine the approximate copy number of MAP organisms in a test sample. Digital (third-generation) PCR, is expected to be more quantitative, more precise and predict absolute numbers of shed bacteria in feces and therefore be useful to validate other tests and biomarkers as has been studied for *Mycobacterium tuberculosis* (Devonshire et al., 2016). microRNAs circulating in blood may provide another a promising method to detect MAP infection (Shaughnessy et al., 2015), but this has not been confirmed.

The effect of MAP genotype on disease progression, shedding, immune responses or generation of other biomarkers is not well characterized (Table 1). A longitudinal follow-up of animals by ELISA, IFN-γ testing, fecal culture and eventually tissue culture is needed.

Using proteomic and transcriptomic analyses, several putative biomarkers for early infection with MAP have been proposed (Skovgaard et al., 2006; Seth et al., 2009; Casey et al., 2011; Zhong et al., 2011; You et al., 2012; David et al., 2014a, b; Thirunavukkarasu et al., 2014; Shin et al., 2015). Metabolomic profiling detected MAP infection earlier than other diagnostic methods, with individual metabolites distinguishing infected from non-infected cattle (De Buck et al., 2014). Furthermore, changes in fecal microbiota of MAP-infected cattle may have promise for identifying infected animals during subclinical stages of JD (Derakshani et al., 2015). Analysis of volatile organic compounds emitted during culture may assist in identifying growth and also strain identification (Trefz et al., 2013). These and other emerging diagnostic technologies and approaches have recently been reviewed by Britton et al. (2016). Unfortunately, none of these biomarkers have been properly validated on naturally infected cattle of varying ages or in various stages of infection to reveal their true sensitivity and specificity (Britton et al., 2016) (Table 1).

Routes of transmission

MAP can be transmitted by: 1) ingestion of fecal material, 2) drinking contaminated colostrum or milk, 3) intrauterine infection and, potentially, 4) aerogenic

transmission. The best method to prevent transmission is expected to be limiting exposure of calves to adult feces by systematic separation of adult cows and calves, in combination with good hygiene practices (Marcé et al., 2011).

There are limited data regarding the quantity of bacteria shed by animals in the different infection stages (Marcé et al., 2010; Whittington et al., 2000b), with substantial inter- and intra-animal variability (Whitlock et al., 2000; Crossley et al., 2005). However, recent advances in PCR technologies (digital droplet PCR; Pinheiro et al., 2012) should enable a determination of the exact number of organisms associated with the different modes of transmission (feces, milk, colostrum) for all different stages of infection (susceptible/uninfected, transiently infectious, latently infected, infectious, resistant stages) (Table 1). This knowledge would be especially meaningful if in parallel the corresponding infectious dose and the corresponding infection risk associated with the various transmission routes were determined for animals of all ages. However, it is important to note that single inoculations may be a poor model of natural infection as the frequency of exposure might be an important factor in transmission. Typically, several or consecutive-day inoculations are used (Hines et al., 2007; Begg et al., 2010). However, a trickle dose (limited numbers of organisms) was highly effective in causing intestinal infection (Eisenberg et al., 2011), suggesting that more complex dynamics play a role during natural infections and need to be taken into account for a better understanding of transmission and infection.

Colostrum

Milk and colostrum can be contaminated with MAP, either through fecal contamination of teats or shedding from the udder (Sweeney et al., 1992; Streeter et al., 1995; Stabel and Goff, 2004). In a Danish study, calves that received colostrum from multiple cows had 1.24 times the odds of testing MAP ELISA-positive as adults compared to cows that only received colostrum from their dam (Nielsen et al., 2008). In contrast, the risk of infection from ingestion of colostrum was recently challenged in a cohort study concluding that MAP-contaminated colostrum did not increase the risk of MAP shedding in calves up to two years of age (Eisenberg et al., 2015a). This reveals a knowledge gap regarding transmission and certainly the relative contribution of some transmission routes (Table 1).

A reduction of within-herd prevalence of MAP infection may be achieved by pasteurizing colostrum to reduce MAP transmission. Heat treatment reduces the number of viable MAP bacteria in milk (Lund et al., 2002; Eltholth et al., 2009). However, most studies used huge numbers of bacteria, spiked milk (not naturally infected), milk instead of colostrum and a hot water bath, not a commercial pasteurizer. Therefore, the current knowledge is insufficient to answer the question whether on-farm pasteurization using commercial pasteurizers can effectively reduce the number of viable MAP bacteria in colostrum (Table 1).

Calf-to-calf

Cattle farmers typically try to interrupt fecal-oral transmission by implementing best-hygiene management practices. In many studies, there were associations between

specific management practices and the likelihood of animals being infected with MAP (e.g. Nielsen and Toft, 2011); however, many questions remain unanswered. In particular, the potential risk of calf-to-calf transmission is largely overlooked in JD prevention and control programs; only 1 of 8 MAP modeling studies included calf-to-calf transmission (reviewed by Marcé et al., 2010) (Table 1). Although many researchers associate a low risk with this potential transmission pathway, based on the assumption that calves will not shed MAP in their feces (Groenendaal et al., 2002; Marcé et al., 2011), there is strong evidence of calves being infected by other calves (Wells and Wagner, 2000; Benedictus et al., 2008; van Roermund et al., 2007). One reason for these apparently discordant results is the difficulty in establishing an exposure-disease relationship, due to the delayed clinical onset of JD (Collins, 1996). Benedictus et al. (2008) reported that calf-to-calf transmission occurs, and that contact with infectious animals increases the likelihood of calves being infected with MAP, which supports previous data (van Roermund et al., 2007). Unfortunately, this study (Benedictus et al., 2008) was done on a single farm and outcomes were measured long after exposure. Consistent with some studies (Bolton et al., 2005; Weber et al., 2006b; van Roermund et al., 2007; Santema et al., 2012), in a recent challenge experiment on age- and dose-dependent susceptibility (Mortier et al., 2013), 47% of calves with a proven MAP infection shed the bacterium at least once from 2 to 6 mo after infection (Mortier et al., 2014c). In a recent study, co-mingling MAP-infected and non-infected calves for 3 mo resulted in infection of 50% of the naïve calves (Corbett et al., 2017). It was estimated that under the circumstances of the study, one MAP-infected calf on average infected 3 other calves. Additionally, on 17 MAP-infected dairy farms in Alberta, Canada, 3% of calves were shedding the bacterium and on 9 of these

farms, MAP-positive environmental samples were collected from young stock pens (Wolf et al., 2015b). Due to acidified milk feeding and automatic milk feeders (Barkema et al., 2015), many dairy calves are group-housed both before and after weaning. Consequently, there is a need for longitudinal studies quantifying the risk of MAP transmission among calves by measuring MAP shedding, environmental contamination, and tissue-levels in naturally infected calves (Table 1). If calf-to-calf transmission is deemed important, JD prevention and control programs will require modifications.

Intra-uterine

Some calves from MAP-infected cows are infected at birth (reviewed by Whittington and Windsor, 2009). Intrauterine infection is more likely in cows with clinical JD (Whittington and Windsor, 2009), and may be lower in herds with a low MAP prevalence (Adaska and Whitlock, 2012). Most studies that determined the proportion of intrauterine infection with MAP recovered fetuses of MAP-infected cows at various stages of pregnancy (Whittington and Windsor, 2009). Since many of these fetuses were recovered prior to term, the proportion of calves born with a MAP infection is undoubtedly underestimated. Additionally, it is not known if or when intrauterine infected calves will start shedding, how infection progresses, and the nature of the immune response, compared to calves infected orally soon after birth (Table 1). The rate of 15% intra-uterine infections may have been over-estimated as a new analysis suggested it may only be 4% (Mitchell et al., 2015).

Intrauterine transmission from dam to fetus appears to be more common in red deer than in cattle and sheep, with 90% of clinically affected hinds having an infected fetus (van Kooten et al., 2006). In another study, MAP was isolated from 78% of fetuses from 18 subclinically infected seropositive red deer hinds (Thompson et al., 2007). By contrast, MAP was isolated from only 39% of fetuses from clinically affected dairy cows and 9% of fetuses from subclinically infected cows (Whittington and Windsor, 2009). In sheep, intrauterine transmission is thought to occur in <10% of infected ewes. Intrauterine transmission of MAP has also been detected in free-ranging red deer and chamois (Deutz et al., 2005). As in cattle, MAP infected colostrum and milk may cause pseudo-vertical transmission in deer and other wildlife.

Environment

If a cow is shedding MAP in the feces, her manure is infectious and can remain so for at least 1 y (Whittington et al., 2004; Whittington et al., 2014). The proportion of environmental manure samples that are culture-positive increases with an increasing prevalence of MAP-infected cows (Wolf et al., 2015a). In sheep, shedding is dose- and age-at-infection dependent (McGregor et al., 2012). However, sheep of all ages and exposed at all doses are equally likely to be colonized by MAP, although the severity of histopathological lesions was strongly determined by age at exposure (McGregor et al., 2012). These findings stress the role of transmission at pasture, especially for young animals.

There is a need for more research to investigate the role of environmental transmission taking into account the survival characteristics of MAP and the contact structure between animals in a herd (Table 1). Survival of MAP in the environment has been suggested to depend on many factors, including soil pH, fecal content, concentrations of macro and micro-nutrients (e.g., Fe, Mo, and Cu), temperature, and exposure to sunlight (reviewed by Elliott et al., 2015). No viable (culturable) MAP was detected after 2 mo of anaerobic digestion at a farm-scale biogas plant (Slana et al., 2011). MAP was present in settled dust samples on dairy farms under both experimental and field conditions (Eisenberg et al., 2010a,b). Although current JD prevention programs do not consider dust, it could be a fomite and facilitate transmission (Corner et al., 2004) (Table 1).

The results of many of the studies on survival in the environment are very difficult to generalize to different environmental circumstances (Elliott et al., 2015) and MAP strain types (Whittington et al., 2004) (Table 1). Secondly, most studies were done as an experimental model and not as a field study. Also, because it is not clear what dose of viable MAP will lead to infection in animals of different age groups, it is not clear whether ingestion of the surviving concentration of MAP bacteria would actually lead to a MAP infection. Additionally, it is not clear whether these factors only influence survival and/or virulence of MAP bacteria, or whether there might also be an influence on the host, through (in)direct effects of these factors on the immune system (Lugton, 2004). Finally, management practices relevant to the transmission of MAP may be associated with environmental factors such as soil type, exposure to sunlight, and humidity (Table 1).

Within-herd transmission

Understanding the routes of MAP transmission between cattle is very important for effective control of the disease. The best-established transmission route of MAP is oral uptake of bacteria by susceptible young stock, via colostrum, milk, water or food contaminated with feces from MAP-shedding animals (Sweeney, 1996; Benedictus et al., 2008). In addition, intra-uterine transmission has been described (see above).

Although isolation of adult cattle from young calves has an important role in prevention of JD (Groenendaal et al., 2002), after 20 y of management and hygiene measures to prevent MAP transmission in a dairy herd, complete eradication was not achieved, implicating other non-identified and therefore non-controlled transmission routes (Table 1). In sheep, experimental intratracheal introduction of MAP caused infection (Kluge et al., 1968), whereas intestinal infection occurred after exposure to MAP-containing aerosols given intratracheally and intranasally (Eisenberg et al., 2011). Since MAP has been detected in bioaerosols on dairy farms, transmission of MAP by bioaerosols should be further studied including estimations of the relative contributions of the various transmission routes identified. Additionally, there is evidence that infection of non-lactating heifers or adult cows can occur (e.g. Fecteau et al., 2010), likely when infection pressure is high.

All existing recommendations (McKenna et al., 2006a) for decreasing the risk of new infections of MAP in a dairy operation are meant to reduce infection rates in calves

by decreasing contact with adult cows. Regardless, a better understanding of transmission and increased testing should improve disease control (Table 1).

Between-herd transmission

Introduction of infected animals is the most important route of transmission of MAP between herds (Rangel et al., 2005). Frequent cattle purchases from other herds without knowledge of their disease status increased the risk for MAP culture-positive environmental samples (Wolf et al., 2016). Although testing animals pre-purchase will prevent introduction of positive animals, the long incubation period will result in many false-negative cattle. Thus, herd-level testing of the herd of origin in a certification-and-surveillance program (Weber et al., 2004; Weber et al., 2006a) is likely to be more effective in reducing the risk associated with trade of cattle between herds. Although MAP can survive for a long time in water (Elliott et al., 2015), the role of transmission through surface and drinking water is not known, and there are many unknowns about the role of transmission from other ruminants and wildlife to domestic ruminants (see below) (Table 1).

Between-species transmission

Cross-grazing sheep and deer reduces the risk of clinical paratuberculosis in deer because the sheep strain of MAP is less pathogenic for deer than the cattle strain (Heuer et al., 2012; Verdugo et al., 2014a). Modeling exercises have shown that the similarity of

strain types isolated from beef cattle and deer was 3-fold greater when direct contact between these species was considered compared to a scenario that ignored the contact structure. Transmission would be expected to go in both directions between co-grazed animals, thus grazing infected deer with sheep or cattle puts both species at risk. In the UK, the presence of farmed deer increased the risk of reporting clinical JD in dairy cattle kept on the same farm (ORs ranged from 15 to 209; Cetinkaya et al., 1997).

In many areas, there is co-grazing of wildlife and domestic livestock, which may allow cross-infection to occur under specific conditions (high animal density, neonates in population, etc.). In an alpine region in Italy, MAP-infected ibex are sympatric with seropositive cattle (Ferroglio et al., 2000). In Spain, cattle, sheep, and goats share pastures and waterholes with herds of fallow deer in which paratuberculosis has been diagnosed (Balseiro et al., 2008). In Germany, it appears that feral and farmed animals are reservoirs for specific MAP genotypes (Fritsch et al., 2012). Although other studies failed to demonstrate significant transmission between wildlife and livestock, false-negatives can be common. It is important to keep in mind that many wildlife studies have limited observations and with that, limited power (Table 1). Also, there is no uniformity in MAP-diagnostics which makes it challenging to compare studies from around the world (Table 1).

Role of wildlife

A better understanding of the epidemiology of MAP in wildlife is essential for implementing effective disease prevention and infection control programs for livestock.

MAP has been recovered from a wide variety of wildlife species worldwide (with or without signs of JD). Mostly it has been found in wild ruminants (reviewed by Mackintosh and Griffin, 2010); however, other species such as lagomorphs may have an important role in MAP transmission (reviewed by Hutchings et al., 2010). Regardless, much remains to be learned about the distribution of MAP among free-ranging wildlife populations, impacts of infection on the health of these populations, potential for these populations to act as reservoirs for MAP, and the extent of MAP transmission between wildlife and livestock in various environments (Table 1).

For most wildlife surveillance efforts, resources are scant. Current methods that reduce the cost of diagnostic testing, such as pooled fecal culture, have only been validated for cattle (van Schaik et al., 2007). Commercially available ELISA tests require validation prior to their use for MAP surveillance in specific wildlife species (Pruvot et al., 2013). Furthermore, it is difficult to sample wildlife populations, particularly to obtain high-quality random samples, which negatively affects the quality of diagnostic results.

Although it has never been quantified, transmission from wildlife is considered a very low risk. Regardless, the livestock industry has opportunities to mitigate the likelihood of inter-species interactions; covering feed storage, keeping wildlife away from forage feeders and other attractants on the farm, and fencing ponds reduced the frequency with which wildlife visit livestock premises (Van Campen and Rhyan, 2010). The livestock industry could be motivated to pro-actively keep wildlife away from livestock if there were well-documented financial benefits. There is limited information available on disease introductions of endemic pathogens through wildlife (Table 1), and most published models focus on introductions of emerging and notifiable diseases with

huge financial impact (e.g. tuberculosis and foot and mouth disease).

The main limitation in considering the role of wildlife species is understanding the circulation of MAP at the livestock-wildlife interface and identifying elements that allow certain wildlife populations to maintain MAP infection and potentially act as a reservoir for livestock. Certainly, it should not be assumed that sympatric wildlife and domestic cattle populations always exchange infection (e.g. Whittington et al., 2001). Increased knowledge on these aspects of the epidemiology of MAP would contribute to our understanding of infection dynamics and may improve JD control programs (Table 1). Unfortunately, with current limited funding opportunities for wildlife-focused studies, as well as the challenges of designing appropriate multidisciplinary studies, these answers might not be easily generated.

Studies to date investigating the possibility of interspecies MAP transmission involving wildlife have focused simply on the presence or absence of shared strains. None have analyzed the degree, directionality of transmission, or incidence of infection caused by spillback or spill-over. Therefore, a genotyping scheme with an appropriate level of discriminatory power, implemented within a well-designed sampling scheme, is essential for reliably investigating the role of wildlife in the epidemiology of MAP (Fritsch et al., 2012) (Table 1).

Susceptibility

Age

Dairy calves are exposed to MAP by the manure from infected adult cattle that shed the bacteria and contaminate water and feed (McKenna et al., 2006a). Greater permeability of the neonatal intestine facilitated MAP entry (Sweeney, 1996), whereas there was increased resistance with age due to repeated exposure to the organism (Delgado et al., 2013) and the dilution effect of the growing rumen (Windsor and Whittington, 2010). However, in an experimental infection trial with 50 calves inoculated at 2 wk, 3, 6, 9, or 12 mo of age, calves were equally susceptible to infection with MAP up to 1 y of age, based on antibody production, fecal shedding, IFN- γ response, pathology, and tissue culture (Mortier et al., 2013; Mortier et al., 2014a; Mortier et al., 2014b; Mortier et al., 2014c; Mortier et al., 2015b). Additionally, infection of non-lactating heifers (1 to 2 y old) occurred when grazing on pasture contaminated with MAP (Fecteau et al., 2010). Therefore, although susceptibility of MAP infection clearly decreases with increasing age (Windsor and Whittington, 2010), control programs should, depending on the purpose of the program, consider including cattle of all ages (Table 1).

Although likely animals of all ages can be infected with MAP, age at infection seems to affect immune responses, fecal shedding, and lesions at necropsy. Calves inoculated at 3 mo of age shed more frequently, had a more robust humoral immune response, and more severe lesions at necropsy than calves inoculated at an older age, or at the same age but with a low dose (Mortier et al., 2014a; Mortier et al., 2014b). The cellular immune response was less marked in calves inoculated at 2 wk of age than calves inoculated later in life (Mortier et al., 2014c), consistent with the need for a strong cellular immune response to confer better protection against infection (Stabel, 2006). In sheep, age of exposure also strongly affected the outcome of MAP infection, with lambs

infected earlier in life starting to shed sooner, having more severe pathology and higher mortality rate (McGregor et al., 2012).

A strong initial cellular immune response, possibly in combination with humoral immunity, appeared to be key to controlling progression of JD (Stabel, 2006). Therefore, infection at a young age when the immune system is still immature and less effective (Chase et al., 2008) generally caused more severe lesions. Therefore, age at the time of infection affected the consequences of MAP infection. In a study analyzing shedding patterns in naturally infected cows, it was clear that the majority of studied cows never developed high shedding levels (Mitchell et al., 2015). Those that did, typically never reduced their shedding level to low or no shedding. Cows that eventually became high shedders had a pattern of continuous shedding. In contrast, cows with an intermittent shedding pattern had a low probability to ever become high shedders. In addition, cows that start shedding at a younger age (less than three years of age) have a lower hazard of becoming high shedders compared to cows starting to shed at an older age. These data suggest the presence of three categories of immune control. Cows that are intermittent shedders have the infection process under control (no progressive infection). Cows that start shedding persistently at a young age partially control the infection, but eventually will be high shedders (slow progressive infection), whereas cows that start shedding persistently at an older age cannot effectively control the infection and become high shedders rapidly (Mitchell et al., 2015). However, little more is known about factors influencing the course of MAP infection.

Heritability of susceptibility and resistance

Quantitative genetic studies predominantly performed in Holstein-Friesian cows have demonstrated that genetics play an important role in susceptibility to JD, and their heritability estimates indicate that the genetic basis of JD is likely a multigenic trait (Koets et al., 2000; Gonda et al., 2006). Channel Island cattle breeds are 1.4 to 8.3 times more likely to test positive for MAP compared to other dairy breeds (Cetinkaya et al., 1997; Norton et al., 2009; Sorge et al., 2011). Outbreaks of JD occur in beef herds, but prevalence of MAP infection is much lower than in dairy herds. *Bos indicus* purebreds and crossbreeds have odds ratios 17-fold and 3.5-fold greater than *Bos taurus* breeds for positive ELISA results (Roussel et al., 2005). Despite likely differences due to management (intensively farmed housed dairy cattle vs. extensively beef cattle kept on large pastures), the lower prevalence in beef cattle may also be the result of lower genetic susceptibility of some beef cattle breeds (Table 1). Genetics as an approach to disease control is an emerging discipline. Identifying the genetic basis of the lower susceptibility will provide possibilities for selection for resistance to MAP infection in dairy and beef cattle, as has been done with Red deer (Dobson et al., 2013). While genetic improvement for disease resistance is slow, the results are permanent (Kirkpatrick and Shook, 2011; van Hulzen et al., 2014). There is good evidence in a range of ruminant species for genetic influence on susceptibility to mycobacterial infections (Kirkpatrick and Shook, 2011).

Heritability of test-positivity for MAP infection in cattle ranges from 0.041 to 0.159 (Koets et al., 2000; Gonda et al., 2006; Hinger et al., 2007; Attalla et al., 2010; van Hulzen et al., 2011) indicating that part of the variability in response to exposure in the

population is due to genetics (Table 1). Multiple recent studies also clearly suggest that susceptibility to MAP infection is multigenic or polygenic. The effect of genetic polymorphisms in candidate genes was recently reviewed (Kirkpatrick and Shook, 2011) as well as linkage analysis of genetic susceptibility using genome-wide association studies (GWAS; Purdie et al., 2011). Limited congruence between studies was attributed to: 1) definitions of case and control; 2) phenotypic data recorded (tissue culture, fecal culture, blood ELISA, milk ELISA); and 3) variability in diagnostic methods used between cattle at the same stage of infection.

The host response to MAP can be categorized as susceptible, resistant or tolerant. Attempts to locate loci associated with resistance to paratuberculosis have proven to be more challenging than finding loci associated with susceptibility. A major problem for investigating the influence of genetics on susceptibility against MAP infection is the difficulty in accurately classifying susceptible, resistant or tolerant animals. For example, it is yet unknown whether selection against ELISA-positivity results in offspring more resistant to (progression of) the infection, or in offspring that is unable to mount an ELISA response given infection.

There is also an inherent uncertainty in phenotypes, due to the low sensitivity of diagnostic tests for MAP infection. Heritability estimates for resistance to JD are higher when fecal culture is used versus ELISA (Kupper et al., 2012). Possible genetic influences on host immunological responses could be a confounding factor (Table 1).

A comprehensive GWAS study (Settles et al., 2009) identified genetic loci associated with 4 phenotypes: presence of MAP in the tissues, presence in feces, presence in both tissues and feces, and presence in tissues but not in feces. Based on identification

of loci associated with these groups, distinct loci may be important to specific stages of the disease. As different genes are associated with different steps in the infection and disease processes, it is expected that different diagnostics will identify different susceptibility genes corresponding to the observed phenotype. Misclassification can be avoided to some extent by parallel test interpretation.

So far, the IFN-γ assay has not been applied in genetic linkage studies. While it is debatable whether a positive test corresponds with active MAP infection or is only evidence of exposure, it is likely that, in combination with other diagnostics, this additional information would help identify different phenotypes, particularly the resistant type (Table 1). Ideally, other biomarkers (e.g. transcriptomics) would be used to identify susceptible animals. For this purpose, biomarker outcomes need to be analyzed with knowledge of the genetic make-up of exposed animals.

Consolidation of marker-assisted breeding approaches for protection of animal populations against paratuberculosis is the ultimate goal of genetic studies. Animal selection based on marker-assisted breeding might lead to cattle populations with enhanced disease resistance and favorable vaccine responses by stimulating protective immune responses (Fisher et al., 2011). However, before these markers can be used to guide selection, undesirable genetic linkages need to be identified. Such a counterproductive linkage occurred when high milk production was genetically associated with slightly increased susceptibility to MAP (Shook et al., 2012). In addition, genetic linkage studies can be expanded from identifying direct associations with susceptibility to infection to associations (e.g. likelihood of progression to clinical

disease, specific responses to vaccination, or tendency to progress to supershedding) (Table 1).

It is not surprising that studies of genetic influence on JD susceptibility of cattle have not had consistent outcomes (Table 1). Apparent causes include size of the study, population structures, markers used and case definitions. Because cattle populations often have a high level of relatedness, the hidden presence of closely related animals in a sample would cause an *a priori* unequal distribution of allele frequencies between cases and controls, which could inflate the rate of false-positive associations between trait and marker (Minozzi et al., 2012). This calls for collaborative cross-border experimental designs, use of appropriate statistical models when analyzing genetic data, and awareness of the genetic structure of the population under study. An initial combined analysis of 2 distinct populations, in which different phenotypic definitions were used, resulted in discovery of novel putative genetic markers for susceptibility (Minozzi et al., 2012).

Future case-control studies should consider limitations of diagnostic tests and lack of knowledge on the family structure of the study objects. However, alternatives to case control studies exist and involve cohort study design (classifying animals according to genotype), with disease outcome being determined after a period sufficient for signs/tests of disease to become apparent and be accurately determined in a longitudinal study (Purdie et al., 2011). Regardless, large numbers of animals are needed to detect small differences in susceptibility due to specific gene loci.

Now that multiple SNPs have been associated with susceptibility to MAP infection, a series of follow-up experiments are important next steps, the so-called post-GWAS functional characterization of susceptibility variants. Detailed molecular studies

to determine the function of the specific nucleotide substitutions mechanistically should follow. Next, experimental infection trials with specific genotypes could be envisioned which would investigate actual effects on infection dose, susceptibility age, diagnostic outcomes, immune responses, immune cell profiles, etc. (Table 1).

Much effort has been spent identifying a connection between host genotype and susceptibility to MAP infection. However, a portion of the variation in disease manifestation and progression might also be correlated with different MAP genotypes (Table 1). Perhaps specific MAP genotypes associate with a specific host genotype. However, such multilevel genotype linkage analyses have not been done.

Vaccination

The Gudair® vaccine has been widely applied in Australian sheep herds and has become the dominant JD control practice. Using this killed vaccine reduced prevalence of MAP infection and fecal shedding, and mortality in Australian sheep herds considerably (Reddacliff et al., 2006; Dhand et al., 2016). This vaccine does not prevent MAP infection, and can therefore not be used on its own to eradicate MAP infection. In contrast to sheep, in cattle no effective vaccine is available, and the lack of an efficacious vaccine that protects against infection with MAP is hampering control programs. Existing JD cattle vaccines can reduce clinical impacts of infection, including sometimes reduced shedding, but they do not prevent infection. Additional obstacles are interference with tests to identify animals infected with other mycobacterial species (e.g. *M. bovis)* and that they can cause severe reactions at the injection site (Kalis et al., 2001). Therefore, there is

clearly a need for better vaccines in addition to improved diagnostic tests for JD (Table 1).

Ideally, a MAP vaccine would protect against infection, keeping negative herds MAP-free and safeguard young and susceptible animals in high-prevalence herds. Arguably, such an ideal vaccine could be live attenuated, can be administered orally, is sufficiently virulent to trigger protective cell-mediated immune responses, protects exposed animals against infection at the tissue level, is cleared relatively quickly from the vaccinated animal, can be differentiated from wild type strains, is not spread to other animals, protects against homologous and heterologous strains, and generates immune responses that can be differentiated from *M. bovis* infections and ideally also from natural MAP infections (with appropriate diagnostic tests).

Current vaccines may partially reduce infectiousness or shedding load, prolong the latent period of infected animals, slow progression from low- to high-shedding, or decrease the cumulative incidence of clinical JD cases; however, they are not effective in preventing infection (Kormendy, 1992; Wentink et al., 1994; Kalis et al., 2001; Kathaperumal et al., 2008; Rosseels and Huygen, 2008; Romano and Huygen, 2009; Santema et al., 2009; Alonso-Hearn et al., 2012). Bacterin vaccines, subunit vaccines (Koets et al., 2006; Hoek et al., 2010; Thakur et al., 2013; Faisal et al., 2013) and vector vaccines (Roupie et al., 2012; Bull et al., 2014) have also been created, but have been less effective than expected. Their inability to prevent establishment of infection leaves the potential for infected animals to break with disease if protective immunity wanes. Subunit vaccines have been reported to provide incomplete protection in murine models (Stabel et al., 2012) or ruminant models (calves and goats) of infection (Koets et al., 2006;

Kathaperumal et al., 2008; Kathaperumal et al., 2009). In addition, other subunit vaccines (Gurung et al., 2013; Facciulo and Mutharia, 2014; Johnston et al., 2014) are being proposed as having potential to prevent infection. Most vaccines have been tested in a preventive pre-exposure setting. However, as calves are commonly born into herds with endemic MAP infection and a MAP contaminated environment post-exposure strategies should also be considered (Santema et al., 2013).

Globally, most current vaccines are based on MAP strain 316F formulated in mineral oil adjuvant, either as a live (Neoparasec) or dead strain (Mycopar, ID-Lelystad, Gudair, Silirum). Only Mycopar is licensed in the US and used on only 5% of US dairy operations (Cho et al., 2012) and under strict control of local veterinary authorities, as vaccinated cattle are more likely to be false-positive on a standard bovine tuberculosis test (Muskens et al., 2002). The single intradermal tuberculin test is still the most widely used tuberculosis diagnostic test in cattle (Bastida and Juste, 2011). However, modification of the test, whereby 2 sites are injected with either tuberculin from *M. bovis* or MAP and a difference in reactivity is recorded, has already been shown to solve the *M. bovis* interference problem in the vast majority of cases. This comparative intradermal tuberculin test has been available for many years and is an official test according to OIE and EU legislation (Bastida and Juste, 2011). So, false-positive *M. bovis* detection can be eliminated when next-generation vaccines are accompanied by compatible diagnostics (Table 1).

A critical aspect in development of MAP vaccines is the protective immune responses they are supposed to elicit. The precise nature of a protective immune response is still to be determined (Table 1). An advantage of live-attenuated vaccines (LAV) is that

they will stimulate both cell-mediated and humoral immune responses (Park et al., 2011; Faisal et al., 2013). Cell-mediated immune responses have been associated with protection (Stabel et al., 2011; Settles et al., 2014), and therefore it has been postulated that if a LAV MAP vaccine could drive the immune response to a proinflammatory Th1 profile and prevent a shift to the humoral Th2 response, it might be more effective in delaying disease progression (Coussens, 2004; Stabel et al., 2011). In sheep, specific lymphocyte subsets play a role in protecting against MAP infection, including sheep vaccinated with killed vaccine (de Silva et al., 2015). Although LAV induced strong protection in a mouse model (Ghosh et al., 2015), in a goat model they showed no protective efficacy in some studies (Hines et al., 2014; Park et al., 2014) and strong reduction in shedding in a recent other study (Shippy et al., 2017); they have apparently not been tested in cattle.

The appropriate level of attenuation of LAV strains has not been established (Table 1). However, it is clear from past experiences that these models should not be restricted to *in vitro* experimentation or mouse models (Bannantine et al., 2014). Furthermore, single knock-out strains may not be optimal vaccine candidates because they may not be attenuated enough to stimulate the protective immune response without causing disease. Although a 1-gene knock-out (KO) might yield a strain that cannot persist in the animal and/or environment and therefore not pose an infectious risk to spread and cause disease, a second attenuation should be introduced to eliminate the manipulative mechanisms that inhibit or counteract protected immune responses. So, a vaccine strain should not only trigger protective immune responses but also avoid immune modulation pathways to be activated, which will allow a wild type (WT) strain

to establish an infection and ensure that the immune evasion strategies of MAP are rendered neutral.

Selection of the first type of essential gene can be done by screening transposon mutant libraries for an optimal LAV (Rathnaiah et al., 2014; Wang et al., 2014), ideally in a ruminant host. However, selection of the second type of KO requires further study of pathogenesis in *in vitro* and *in vivo* models. We need a better understanding of the immune evasion and immune modulation strategies that MAP clearly is capable of (Arsenault et al., 2014) (Table 1). Due to these immune evasion properties, MAP can subvert both the induction of acquired immune responses and cell-mediated responses. Reasons for these are potentially twofold: first, because the LAV vaccine will have retained these immune evasion properties and therefore elicit inadequate immune responses; and secondly, because an infecting WT strain will be able to subvert immune responses generated by a vaccine. A future protective vaccine may need to prevent or counteract these evasion mechanisms.

Diagnostic aspects of vaccination

Differentiation of Infected and Vaccinated Animals (DIVA) is an important consideration. When using a subunit vaccine approach, DIVA can be obtained as has been shown in cattle vaccinated with a subunit vaccine against MAP (Santema et al., 2009). None of the three currently commercially available killed vaccines or LAV lend themselves well to DIVA, although engineered vaccine strains might be better. Complementary diagnostics will have to be developed to incorporate DIVA in an

integrated platform of new diagnostic and vaccination strategies, particularly to differentiate *M. bovis* infections from JD vaccination (Table 1). Next-generation diagnostics for MAP infection will likely measure early cell-mediated immune responses. Immune responses against this positive marker of extraneous nature would clearly demonstrate that animals were vaccinated (Table 1). A negative marker, being an immunodominant antigen that is 'deleted' from the vaccine strain, could be implemented in novel *M. bovis* diagnostics next to antigens specific for *M. bovis* (e.g. ESAT-6/CFP-10 and Rv3615c) (De Val et al., 2012). Thus, the problematic interference with *M. bovis* testing could be completely mitigated with a marked vaccine and compatible diagnostics.

Following complementary diagnostics for DIVA purposes, new biomarkers that act as correlates of protection and/or that indicate lack of protection are necessary for screening, development, and testing of novel MAP vaccine candidates (de Silva et al., 2015). So far, good correlates of protection have remained elusive.

From exercises modeling impacts of imperfect MAP vaccines, it was concluded that vaccination should be integrated into a comprehensive control program that includes test-and-cull intervention and improved calf rearing management (Bush et al., 2008; Cho et al., 2012). Cost-benefit analysis of vaccination against MAP in dairy cattle was performed (Groenendaal et al., 2015). Vaccination was beneficial by reducing the frequency of heavy shedders and clinically affected animals. A meta-analysis also concluded that vaccination against MAP is a valuable tool in reducing MAP contamination risks and reducing or delaying production losses (Bastida and Juste, 2011).

Newly developed vaccines will need to be well-characterized to fully understand their risks and benefits. For example, the risk of shedding in vaccinated cattle needs to be

investigated, as was done in sheep vaccinated with the Gudair vaccine (Windsor et al., 2014).

The success of a LAV vaccine will depend on which MAP strain is selected to generate the vaccine (~parent strain). Vaccine strains will likely have to be sufficiently homologous in antigen composition with the majority of field strains. In India, a vaccine based on a regional strain variant was more effective than a commercial JD vaccine (Singh et al., 2013). In our view, there should be a focused effort to characterize the diversity of MAP strains (Table 1). Genotypic and phenotypic variation needs to be investigated to derive quantitative and qualitative understanding of this diversity around the world.

Uptake

Nearly all JD prevention and control programs worldwide are voluntary and their success depends on enrollment and retention, along with sufficient uptake of recommended best management practices. To establish high participation rates, JD control programs need to account for farmers' motivators and barriers to enroll and implement recommended management changes (Table 1). In recent years, farmers' "mindset" and its influence on behavior has become an important focus of research (e.g., Derks et al., 2012; Jansen and Lam, 2012; Garforth, 2015; Roche et al., 2015). Farmers' attitudes and beliefs toward the disease and the proposed approach for disease control have important roles in their motivation to adhere to suggested management strategies (Ritter et al., 2017). This relatively new avenue of research has important implications for our approaches to motivating individual farmers to adopt optimal JD management practices.

Farmers' considerations such as improved herd health and concern over consumer health can be important motivators to participate in JD control or certification programs (Kovich et al., 2006; Nielsen, 2011; Roche et al., 2014). External incentives can also be an important driver to control MAP; a decision analysis from the farmers perspective indicated that a milk-price differentiation of only € 0.005/kg milk was sufficient to make enrollment of Dutch dairy farmers in a control program attractive (Velthuis et al., 2006). However, management constraints (e.g. limited time or finances) and the perceived complexity of JD control programs can be critical impediments for uptake of biosecurity measures (Rossiter and Burhans, 1996; Wraight et al., 2000; Sorge et al., 2010), and often farmers prefer to "wait-and-see" how the JD control program works on other farms before they enroll (Ritter et al., 2015) (Table 1).

Several studies reported that farmers rely on various sources to obtain their farm management information and should be approached according to their specific needs to ensure successful knowledge uptake (Heffernan et al., 2008; Jansen et al., 2010; Russell and Bewley, 2011). In particular, herd health veterinarians have been regarded as trustworthy and reliable sources of advice on disease and disease risk management (Ellis-Iversen et al., 2010; Brennan and Christley, 2013). Because of this key role veterinarians play in farmers' management decisions, it is important to employ them as mediators between industry and farmers. However, veterinary practitioners' attitudes towards JD control are often unknown and the extent to which they actively promote enrollment in JD control programs and/or individual on-farm changes to reduce MAP transmission remains unclear (Table 1). Even farmers provided with veterinary advice on JD control often implemented less than half of the suggestions made (Wraight et al., 2000; Sorge et al., 2010). Miscommunication between dairy practitioners and farmers is likely an important cause of the lack of uptake. For example, veterinarians did not assess producers'

expectations sufficiently but provided them with too many, potentially overwhelming, suggestions (Sorge et al., 2010). These apparent gaps in veterinary-farmer communication need to be addressed.

More recent applied research has investigated best practices for communicating with producers and motivating on-farm change, often employing group-based and peer learning approaches. Many of these efforts have emphasized the importance of the veterinarian for making tailored on-farm recommendations, but have also promoted the use of facilitators to better understand and respond to producer mindset. Recent examples from Australia (Kingham and Links, 2012), Denmark (Trier et al., 2012), and Canada (Roche et al., 2015) have yielded promising results for motivating on-farm change towards effective JD prevention and control.

In addition to a lack of knowledge regarding the discussion of JD control with producers, communication strategies used to inform decision makers in government and policy authorities, farmer organizations, and breeding associations are still unclear. Researchers' responsibility is to provide information that enable evidence-based decisions, for example regarding JD certification and trade regulations. Clear communication between researchers, farmer organizations, and authorities creating evidence-based policy will likely increase motivation to initiate and continue control programs and improve policy acceptance and uptake by farmers.

Johne's disease is only one of many fecal-orally transmitted infectious diseases in cattle. Measures to prevent infection with MAP will likely also have positive effects on the incidence of infection with *Salmonella* spp., *Cryptosporidium parvum* and *Cryptosporidium bovis*, *Escherichia coli*, and rota- and corona virus (McKenna et al.,

2006b). Often, the latter bacteria cause more obvious clinical signs than MAP and convince the farmer of the presence and severity of illness. These "cues-to-action" might enhance farmers' openness to improve suboptimal management practices. Therefore, addressing calf health and biosecurity more holistically could help motivate producers that currently do not perceive JD control as high priority (Table 1).

Conclusions

Nearly a century of JD control programs has, particularly in the dairy industry, not resulted in sufficient progress. Except for goats in Norway, no reports can be found of a herd in which MAP infection has been eradicated, and in many countries, herd- and animal-level prevalence has not decreased. As a result, JD continues to cause considerable losses to the livestock industry. The insufficient progress has been the result of gaps in our knowledge about this difficult disease. Research has focused on test development and evaluation, vaccine development, and design and evaluation of management strategies to prevent MAP infection. Many of the knowledge gaps identified are in these areas (Table 1). However, the authors are optimistic that if sufficient progress can be made addressing these knowledge gaps, progress in the control of this insidious disease in the next decades will be better. The introduction of a JD vaccine has made a huge impact in the Australian sheep industry. Development of a JD vaccine with accompanying diagnostic tests that prevents infection and shedding and does not impair tuberculosis diagnostics remains 1 of the most pressing gaps for the livestock industry. Reliably and pro-actively (pre-shedding) identifying infected animals that will very likely

shed the pathogen, potentially involving biomarkers, is another research priority. Susceptibility for MAP infection differs among breeds. Identification of genetic markers that distinguish very susceptible from more resistant animals has the potential to advance JD control. Quantification of the role of calf-to-calf transmission will be necessary to improve cattle control programs. Uptake of JD control programs will improve if these knowledge gaps have been satisfactorily addressed. However, because of the voluntary nature of JD programs, it will still be important to identify factors that motivate farmers to enroll in these programs.

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Table 1. Most important knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection

Area	Knowledge gap
Control programs	- (Long-term) efficacy of control programs - Is eradication of MAP infection in a herd possible?
Prevalence	- Comparison of MAP prevalence over time in the same region - Comparison of prevalence in different regions using the same test (regime)
The pathogen	- Distribution of MAP genotypes - Differences in virulence, pathogenicity, immunogenicity, persistence, transmission, survival outside the host, and host specificity between genotypes - Effect of mixed genotype infections and superinfections
Tests	- Characteristics of tests in a population that reflects the target population - Development of reliable early stage diagnostics - Reliable on-farm tests - Value of detection of immunoglobulin isotypes other than IgG - Most accurate and cost-effective set of environmental samples and sampling interval (dairy) - Best DNA extraction protocols for PCR analyses - How to quantify MAP in tissues, blood, milk, and feces - Validation of biomarkers in naturally infected cattle
Transmission	- Role of MAP shedding young stock - Relative importance of various transmission routes (including transmission through dust and drinking water) - Influences on survival of MAP in soil and on pasture) - Minimal infectious dose for animals of all ages - Consequence and importance of intra-uterine transmission - Shedding pattern of MAP-infected animals, including role of supershedders - Effectiveness of commercial pasteurizers at reducing viable MAP bacteria in colostrum
Role of wildlife	- Validation of diagnostic tests for wildlife - Prevalence of MAP in wildlife - Impact of MAP infections on wildlife health

	- Magnitude of the role wildlife plays as MAP reservoir and in transmitting MAP to farmed animals
	- Economic benefits of reducing MAP transmission from wildlife to livestock
Susceptibility	- Influences on susceptibility and the progression of MAP infection (e.g., age, genetics,..)
	- Genetic heritability of MAP infection
	- Difference in susceptibility among breeds
	- Identification of genetic markers associated with MAP susceptibility, disease progression, and response to vaccination
	- Benefit of the IFN-γ assay in genetic linkage studies
	- Interaction between host genotype and MAP genotype
Vaccination	- Development of a cattle vaccine that limits infection and/or shedding, and does not interfere with diagnostics for other mycobacterial infections
	- Better understanding of immune evasion and immune modulation strategies of MAP
	- Characterization of diversity of MAP strains to select the most appropriate vaccine strain
	- Definition of protective immune response by vaccination
	- Appropriate level of attenuation of vaccine strains to elicit immune response without causing disease
	- Differentiation between infected and vaccinated animals
Uptake	- Influences on farmers' motivation to enroll in (voluntary) disease prevention and control programs and implement recommended management strategies
	- How to overcome the "wait-and-see' mindset
	- Veterinary practitioners' role in promoting control program participation including communication
	- Potential of approaches that address Johne's disease in combination with other fecal-orally transmitted diseases