No Guarantees with Johne's Disease: Use of common and novel diagnostic methods

Jeroen De Buck

Department of Production Animal Health, University of Calgary, Alberta, Canada
Email: jdebuck@ucalgary.ca

Take Home Messages

- Keep the purpose of testing in mind when choosing Johne's disease (JD) diagnostics.
- The usefulness of current JD diagnostics increases with frequency of testing.
- Inclusion of young stock and calves in testing strategies is recommended.
- Novel JD diagnostics will come from pathogenomic methods to detect characteristic gene expression, protein biomarkers, metabolites, lipids and shifts in the gut microbiome during *Mycobacterium avium* subsp. *paratuberculosis* infection.

General Introduction

Johne's disease (JD) is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). After MAP is ingested by the animal it invades the wall of the intestinal tract. Microscopic and macroscopic lesions inhibit absorption of nutrients through the gut lining, particularly in the small intestine. Furthermore, energy is expended by the animal in mounting an immune response. Subclinical infection with MAP results in lower milk yields, poor reproductive performance, and reduced value of culled cows, thereby reducing profitability of the dairy operation.

Subclinically infected animals will have a lower carcass weight relatively early after infection compared with non-infected animals. This was demonstrated in an experimental infection trial (Roy et al., 2017). This reduced weight was also seen in studies of the metabolites in the blood of these calves, demonstrating signatures of starvation (De Buck et al., 2014). Dairy cows that are milk MAP enzyme-linked immunosorbent assay (ELISA)-positive weigh 10% less at slaughter than milk ELISA-negative cows. Furthermore, if the cow is shedding MAP, its weight is on average 15% less compared with non-

shedders (Kudahl and Nielsen, 2009). These findings clearly demonstrate that infection with MAP will have production-limiting effects, mostly on the subclinically infected animals, but also the herd in general.

■ Purpose of Testing

Diagnostic tests need to serve an actionable purpose. Test results used in control programs should primarily guide decision-makers to take appropriate steps. Most often, the goal of a cost-effective test strategy is the identification of infected animals before they spread the infection while still maximizing the lifetime production of an animal. Purposes of testing primarily include the following: 1) to estimate infection prevalence so that the best course of action can be determined; 2) to minimize financial losses that result from diminished milk production or growth and increased culling rate; 3) to estimate the effects of proposed control measures or evaluate additional measures or management changes; 4) to eliminate infectious cattle to reduce spread of infection, 5) to 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. Individual animals are typically targeted to identify the infectious animals that are actively shedding MAP and to identify the infected animals at risk of shedding MAP.

Existing tests perform reasonably well in detecting advanced stages of JD, although the specificity (true negative rate) of test results may be challenged. Detection of early-stage infection is only relevant for a control program if these animals then become infectious. However, because the sensitivity (true positive rate) of diagnostic tests in calves is generally low, young stock are rarely tested for MAP.

■ Herd-level Diagnostics

Environmental sampling is a quick sampling method to determine the MAP infection status of a herd. However, this sampling method is only sufficiently sensitive in relatively intensive livestock operations such as housed dairy cattle. For example, MAP culture followed by Polymerase Chain Reaction (PCR, a method to make copies of a specific DNA sequence to aid in its detection) on six 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. Culture of pooled fecal samples, most often consisting of pools of five or ten animals, has been extensively evaluated and proven to be relatively sensitive.

Testing of bulk tank milk or a pool using a commercial ELISA is an inexpensive method of herd screening for JD in dairy cattle. 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. This testing identifies herds with moderate to high prevalence. This test is not sensitive to detect low prevalence herds or to monitor subtle changes in within-herd prevalence over time.

■ Late-stage Diagnostics

The titer (indicative of antibodies against MAP) of an ELISA is predictive of the probability of MAP shedding in individual animals. This makes ELISA a highly useful tool in 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. Rising antibody titers help to detect infected animals, and repeat fecal PCR addresses false negatives due to intermittent shedding.

■ Early-stage Diagnostics

Antibody responses are typically not expected in young animals; so, serological tests that overcome the current delay in antibody-based detection of MAP infection are the focus of active research. However, some studies are showing antibody against MAP in samples as early as 70 days after infection (Bannantine et al., 2008) and three months after infection (Mortier et al., 2014a).

Early-stage diagnostics primarily target the initial pro-inflammatory immune responses. The interferon-gamma (IFN-γ; a protein produced by a specific type of white blood cell in response to MAP—it initiates an immune response to MAP) assay detects these responses to early-stage MAP infection but needs improvements and standardization to improve specificity. Specific proteins to increase specificity of T-cell responses (T-cells are white blood cells that attack foreign substances such as bacteria; they also produce IFN-γ) are still being sought. Blood samples must be tested in a laboratory within eight hours for optimal results to ensure T-cell viability. Ultimately, an on-farm test for this early cell-mediated immune response would be most effective. Such an on-farm test might be based on a modification of the existing Interferon-gamma release assay (IGRA), or immune cell markers associated with MAP infection may also be used as a diagnostic test.

Bacterial culture and PCR are well known examples of diagnostics that directly identify the presence of MAP in clinical samples. Here too, the value of ELISA and PCR can be increased by higher testing frequency. Perhaps less known is the use of mycobacteriophages (viruses that infect and kill mycobacteria) to detect mycobacteria causing bovine infectious diseases such as JD and bovine tuberculosis. Sensitive methods have been developed

to take advantage of these phages to only replicate in viable MAP cells. The specificity of the test can be improved by complementary PCR tests because MAP-specific mycobacteriophages have not yet been identified.

■ Young Stock

When producers want to screen their herds for JD, they are advised to test cattle older than 36 months (Collins, 2011), because the current thought is that calves acquire MAP infection at a young age and go through a carrier and a subclinical stage during which diagnostic tests have a low sensitivity. Consequently, young stock is typically not included in herd screening and early shedding or humoral immune responses (an immune response in which B cells, a type of white blood cell, produce antibodies in response to MAP) can be missed. Yet, in calves inoculated at two weeks, or at three, six, nine, or 12 months of age with a low or high dose of MAP, fecal shedding was detected in 61% of the calves (Mortier et al., 2014b), which was followed by an IFN-γ response that peaked at four months after inoculation (Mortier et al., 2014c).

A relatively high proportion of young stock on confirmed MAP-positive farms shed MAP in their feces, indicating that a potential route of transmission is currently being overlooked (Weber et al., 2010; Wolf et al., 2015b). The decision to include young stock in JD monitoring is not straightforward. Benefits of a program that incorporates young stock would include early detection of infectious and potentially infected animals (to prevent new transmissions), environmental sampling (to detect young stock housing as a potential transmission site) and identification of MAP-positive young stock (potential indicator of cow-calf transmission). However, challenges arise when deciding which diagnostic test to use to identify MAP-positive calves, and what to do with the calves identified as MAP-positive. Fecal shedding among calves can be intermittent, changing day-to-day, making detection of infectious calves difficult and highly variable. Furthermore, fecal shedding does not necessarily indicate that a calf has a tissue infection. Therefore, repeated testing, separating positive calves from potentially susceptible pen mates, and continued monitoring would be advised as an alternative option to immediate culling. Methods for surveillance of potentially infectious calves will have an immediate economic impact on producers, resulting from repeated testing and from implementing separate designated housing areas with designated equipment for these fecal-positive animals.

Because shedding, substantial humoral immune responses (antibody production) and IFN-γ responses were detected in inoculated calves, catching these initial host responses before continuous shedding starts could allow early diagnosis and an earlier intervention from control programs.

■ Diagnostic Test Agreement

With the availability of multiple yet imperfect diagnostic options, we must understand how diagnostic tests relate to each other to maximize their applicability. However, diagnostic test agreement between all available diagnostic tests (IFN-γ release assay, antibody ELISA, fecal culture, fecal PCR, gross lesions, tissue culture and histopathology) within a sample population is underreported for paratuberculosis. The low test agreement that is typically observed may be due to pathogenesis (the mechanisms that lead to disease development), such that fecal shedding and antibody production do not necessarily occur simultaneously in a MAP-infected individual. To optimize detection of infected cattle, we must determine the onset of positivity of these diagnostics.

The underlying cause of fecal shedding is unknown; however, several hypotheses link a cellular immune response to fecal shedding. Understanding the interaction of shedding and cellular immune response could improve early diagnosis of MAP-infection.

■ Potential sources of individual variation impacting diagnostics

Calves in a controlled infection trial have responded differently on diagnostic tests. In a challenge experiment, several antibody response profiles were observed (no response, transient response, persistent response (Kawaji et al., 2012) and onset varied for each calf (Mortier et al., 2014a). Furthermore, fecal shedding patterns can be very different for each inoculated calf (Mortier et al., 2014b). Possible explanations for this individual variation are environmental factors, stress level of the calf (such as heat, cold, reduced feed intake on the day of inoculation), nutrition of the calf (e.g., recent meal at the moment of inoculation versus some hours since the last meal might affect MAP uptake) and microbiome (community of bacteria in the gut) of the calf at the moment of inoculation or any time after that. Because experimental infection trials are conducted in a controlled environment, the differences in responses between all calves suggest a genetic basis of this observed variation.

■ What are the 'other' signals that could be picked up from Johne's disease?

MAP is very much a sneaky and stealthy pathogen. It avoids detection by actively hiding from and modulating the host's immune responses. MAP lives inside compartments inside macrophages (a type of phagocyte, which is a cell that detects, engulfs and destroys pathogens) which are located in the tissues

of the intestinal tract. It is hidden from patrolling immune cells by living inside immune cells. From this location MAP further decreases detection and exposure by limiting the presentation of MAP antigens on the surface of these immune cells. This is a dynamic situation whereby macrophages die, release MAP and attract new phagocytes to be exposed to MAP.

Given this situation, what signals can we expect to pick up from these intracellular organisms to help us with diagnosis—signals that can be detected in clinical samples? These samples are typically blood, milk and feces, but could theoretically extend to saliva and urine.

Detection of MAP could be direct by detecting its DNA, by detecting the expression of its genes, by detecting its proteins or other cellular components, or by detecting characteristic small molecules (metabolites) produced by this microorganism. These signals can be detected with corresponding approaches, called genetics, genomics, transcriptomics, proteomics, and metabolomics.

The effects on the host (calf or cow) of the infection, immune responses and inflammation can also generate indirect signals. These signals will reflect changes in immune cell populations, changes in host gene expression and changes in protein biomarker levels. In turn, all these changes impact the levels of metabolites, and profiles of lipids and volatile compounds present in samples.

Less explored are effects of MAP infection and intestinal inflammation on the gut microbiota of the infected cattle. These microbes in the gut sense changes and adapt to new conditions. This is bound to be reflected in shifts in microbiome composition, changes in gene expression by members of the intestinal microbial community and eventually in the proteins that are produced by these members. In turn, these changes will lead to potentially marked changes in metabolites produced by the gut microbiota.

None of the changes outlined above happen in isolation. Gene expression by MAP has the potential to influence the gene expression of host cells. This in turn can affect the environment sensed by the microbiota. In short, the three parties (MAP, host, microbiota) will influence each other as illustrated in Figure 1.

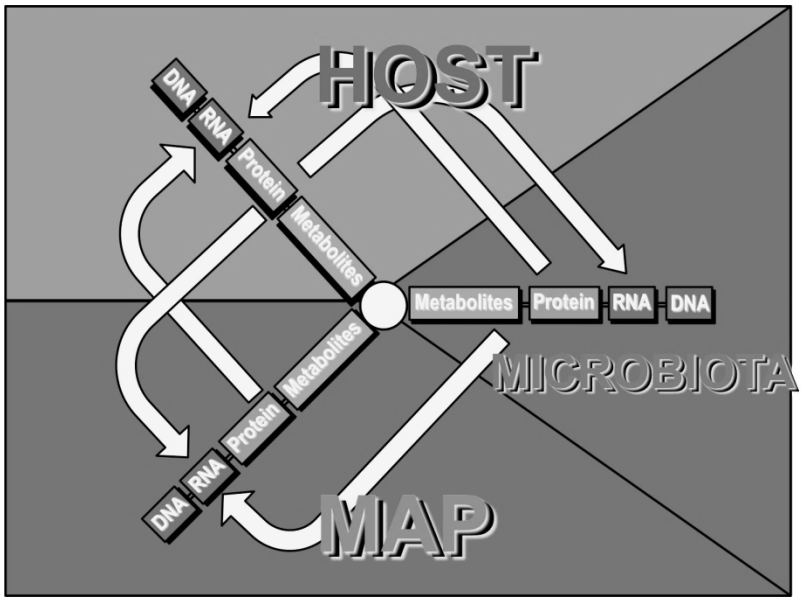

Figure 1. Relationships between MAP, the host, and the host's microbiota

■ How are these signals being used for diagnosis?

The term 'pathogenomics' groups all comprehensive studies of biological molecules such as proteins, DNA, RNA, small metabolites, lipids or carbohydrates, in relation to disease processes. Each of these classes of molecules individually contain information of disease processes. The integration and merging of different pathogenomic technologies is expected to give an even greater power to resolve complex questions about MAP biology and host pathogen interactions in MAP infections. Currently, MAP pathogenomics are applied to discover biomarkers of infection for diagnostic purposes.

In this presentation, an overview will be provided about how state-of-the-art technologies are being used to develop better MAP diagnostics.

Genomics

A large number of MAP whole genomes are now available from different countries. The evolutionary relationship of MAP strains and subtypes have been determined and the genetic diversity and distribution the genotypes have been uncovered. This has helped to ensure that diagnostic targets are present in all MAP strains.

Transcriptomics

Transcriptomics consists of methods to study the gene expression of pathogens or their hosts. Transcriptomic studies have revealed some of the host responses during experimental MAP infections. Characteristic gene expression profiles that can be reliably detected in all infected cattle have the potential to have diagnostic value. Total RNA sequencing (RNA seq) is increasingly being used to study immune responses against MAP, mostly on in vitro cell culture models, but also in naturally infected cattle. New methods are on the horizon to enable researchers to study the transcripts of host and pathogen.

Metabolomics

The growing field of metabolic profiling (i.e., metabolomics) involves identification and quantification of numerous low molecular weight compounds in biological fluid samples. Metabolomics provides a functional alternative or complement to the above-mentioned techniques; it measures chemical phenotypes that are the net result of all activity on the transcriptome and proteome levels. Therefore, it provides a simplified summary of the status of the infected animal. Previously, a Nuclear Magnetic Resonance based metabolomic approach was successfully used to study changes in experimentally infected calves. A follow-up field trial using the same technology will be presented, with emphasis on the experienced challenges.

More recently, infected cattle were also studied by direct lipidomics, the analysis of all different lipids present in a sample (Wood et al., 2018). Results from this analysis indicate that altered availability of choline-containing lipids occurs late in the disease process and is most likely a result of malnutrition and altered biosynthetic capacities of the liver and gastrointestinal tract. Alterations in the bioavailability of these critical structural lipids presumably contributes to the demise of MAP infected cattle. Other metabolomic studies are underway to discover early biomarkers of infection retrospectively on animals that turn positive on common diagnostic tests later on. In this project, age-cohorts from JD-affected farms were studied over a two-year time period during which each animal was periodically tested for MAP infection by MAP-fecal culture, PCR and ELISA.

Metagenomics

Metagenomics consists of technologies to investigate the presence and abundance of all bacterial organisms in a certain environmental or tissue location. Intestinal inflammation is a hallmark of paratuberculosis. However, there is a lack of knowledge regarding impacts of the inflammatory responses on the composition and functional properties of intestinal microbiota. So far,

only one study has tried to determine the pattern of MAP-associated imbalance of intestinal microbiota as a potential biomarker for early detection of infected cattle (Derakhshani et al., 2016). Enrichment and under-representation of certain metabolomic pathways suggest an interplay of the intestinal microbiota and the immune system. Specific changes in composition of the gut microbiome provided potential biomarkers for identifying infected cattle during subclinical stages of JD.

What can we expect in the future?

Using any or all of the above technologies, we need to find biomarkers of infection that are stable over time so that they can be used to reliably detect MAP infection in cattle of any age and any stage of disease. This is particularly difficult as we know that the disease is progressing at all times and the responses or different animals vary in timing, nature and intensity. To accommodate the discovery of biomarkers that can predict the onset of the infectious stage, experimental infection models presenting a natural disease outcome are essential for test development. Candidate biomarkers, identified in experimental infections, will always need to be validated by field testing. Repeated sampling of herds is necessary to validate the outcomes of experimental infections in an unbiased and representative set of samples from infected but not yet infectious animals. We should also focus on biomarkers that give us information about the likelihood of fecal shedding.

The best approach in validating a panel of candidate biomarkers is by creating a sample collection of animals over time. Retrospective analysis of those samples collected from animals that were later confirmed positive by standard diagnostics will allow discovery and validation of early diagnostic methods. Such large scale studies are costly yet necessary to improve JD diagnostics.

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