The specificity of phage testing for MAP – where might it fit into the diagnostic armoury?

Abstract

The current individual tools available for the diagnosis of Johne’s disease are far from suitable to tackle this endemic disease. Culture, PCR and ELISA tests, when used together can be useful in managing the disease in the later stages of infection at a herd level. They are, however, ill-suited to detecting the causative agent *M. avium* subsp. *paratuberculosis* (MAP) at the early stages of infection and at an individual level. Phage technology offers another tool in the attempt to better manage and control this disease. Phage-technology has been demonstrated to rapidly and sensitively detect and specifically identify viable MAP in the milk and blood of cattle. Although in relatively-early stages of development, phage technology offers a strong addition to the armoury of tests used to detect MAP in blood and milk, and may go on to be part of ongoing control measures to reduce the burden of disease to farmers and veterinarians.

Introduction - Johne’s disease

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne’s disease; a chronic infectious granulomatous enteritis of ruminants that is endemic in the UK as well as many countries worldwide. As well as being a serious disease from an animal health perspective, Johne’s disease also places a significant economic burden on farmers.

There are generally four stages of infection for Johne’s disease, which often span several years: silent, sub-clinical, clinical and advanced. Shedding of MAP cells in faeces can occur during the subclinical stages of infection, resulting in transmission on farm via the faecal oral route. MAP can also be shed into the milk of infected animals leading to transmission to newly born animals. Whatever the route of transmission, it is believed that initial MAP-infection occurs within the first few days of life, but clinical signs often do not appear until animals reach 3–4 years of age. This long incubation period before Johne’s disease becomes detectable using conventional tests makes effective control schemes difficult to implement.
Tried and tested: current tools for diagnosing Johne’s disease

Although classical laboratory culture of MAP is considered the gold standard method of detection, in practice it is very difficult to perform because MAP is a slow growing organism that is both difficult and expensive to culture. It can take over 16 weeks to culture the bacterium on laboratory media and there are high failure rates due to the high risk of contamination by faster growing organisms. To counter this problem, samples such as milk, faeces or tissue biopsies are decontaminated using chemicals, but these can also affect the viability of the MAP cells in the sample, thus reducing the sensitivity of culture further. Not only does this inability to routinely culture MAP make the gold standard diagnostic test impractical, it also means that fundamental aspects of the pathophysiology of Johne’s disease are not well understood, which hinders the development of control programmes.

There are a range of immunological-based diagnostics that have been developed to diagnose Johne’s disease. There are many commercial and in-house enzyme-linked immunosorbent assays (ELISA) to detect anti-MAP antibodies in bovine milk, serum or plasma. However there can be issues with cross-reactions occurring if the animals have been exposed to other pathogenic or environmental mycobacteria which may result in false-positive results. Another issue identified is that depending on the age and stage of infection, sensitivity of detection can be as low at 2% in calves to 55% in older cattle. Poor sensitivity of ELISA tests remains a significant problem and is an unavoidable consequence of the chronic nature of the disease. Since commercial ELISA tests are generally only capable of routinely diagnosing Johne’s disease in animals at the later stages of infection, or only during the very late stages of the subclinical phase of infection, they are not really sensitive enough to be used on an individual animal basis to reliably confirm infection. Most control programmes test herds annually, however to compensate for the lack of sensitivity, and the fact that antibody levels in infected animals are known to fluctuate, quarterly testing of individual animals is often carried out, with the pattern of positive test results being used to classify the infection risk status of an animal.
Despite this limitation, ELISA tests are routinely used as part of disease management programmes as the high-throughput format and low cost per sample means herds can be screened very quickly and simply. In addition, ELISAs can be prognostic indicators for reduced milk yield or faecal shedding.\textsuperscript{15}

Molecular detection methods have been developed for the detection of MAP based on detection of signature DNA sequences by PCR amplification (or other nucleic acid amplification technologies) that enables the rapid and accurate detection of these bacteria in a variety of matrices. The most commonly used genetic target is the IS\textsuperscript{900} insertion element found in multiple copies in the MAP genome and is considered to be unique to MAP, although the rare occurrence of IS\textsuperscript{900}-like sequences have been reported in the literature.\textsuperscript{6,7} The fact that IS\textsuperscript{900} is found in multiple copies within the MAP genome makes it an ideal signature sequence as the PCR-based detection will be more sensitive than tests that target single copy genetic elements.\textsuperscript{21}

PCR-based detection is often used when MAP is present in matrices such as faeces or milk to overcome the problems associated with direct culture described previously. However these matrices also contain many PCR inhibitors. Hence, accurate and sensitive detection of mycobacterial DNA by PCR relies on both efficient lysis of the bacilli and DNA purification to remove PCR inhibitors and concentrate DNA.\textsuperscript{10} Efficient lysis is one of the biggest problems when using direct PCR to detect mycobacterial DNA because mycobacteria have a very thick cell wall, which make traditional lysis methods very inefficient, thus reducing the sensitivity of any PCR-based detection event. Where experimentally, nucleic acid amplification technologies can be shown to specifically and sensitively detect MAP, the sample preparation and DNA lysis steps are often inefficient, reducing the power of these technologies. Therefore direct PCR-based detection often require the MAP cells to be present in high concentrations but the reliability of such methods is confounded by the fact that the concentration of MAP cells in a sample also fluctuates and is very much dependant on the stage of infection and bacterial shedding levels.\textsuperscript{2}

\textbf{A new approach: Phage Technology}
Bacteriophage are viruses that infect bacteria and they will only successfully replicate within a viable host. Like all viruses they identify their correct host cells by binding to specific structures (receptors) on the outside of the bacterial cells. Once bound, phage inject their own DNA into the bacterial cell and then take over its machinery to make many copies of themselves before finally producing enzymes that break down the cell wall and cause the cell to break open, releasing new phage particles into the environment (Table 1). Phage have been used for many years to rapidly detect different bacterial pathogens, including members of the *Mycobacterium* genus. Several different phage-based detection assays have been described, such as genetically engineered reporter-phage that produce a fluorescent or bioluminescent signal when they infect their host cells, or phage binding to host cells can be detected using physical methods such as plasmon resonance-based spectrometry (for a full review see Schofield et al., 2012). However more recently the detection of mycobacteria using bacteriophage amplification technology has been developed where the natural life cycle of the phage is exploited to detect its bacterial host.

Bacteriophage amplification technology has been exploited to detect a range of mycobacteria. One of the original uses that was commercialised was to detect *M. tuberculosis* -the causative agent of tuberculosis (TB) - in human sputum samples. The assay was a low cost and simple petri-dish based test that enabled mycobacteria to be detected within 48 h (for review see Rees & Botsaris, 2012). To develop this assay a broad spectrum bacteriophage, D29, was used which is capable of detecting a wide range of mycobacteria, including pathogenic and non-pathogenic species. The ability of the assay to detect many strains, led to the phage amplification assay being applied in the agricultural and food sectors. However, the broad host range of phage D29 was both an advantage and disadvantage where in human clinical samples, mycobacteria in the sputum would be treated in the same way regardless of what species was present. However in food and agriculture settings, many other mycobacterial species, both pathogenic and environmental may be present in the samples. This results in the detection of a range of mycobacteria not of interest reducing the specificity of the assay in this plate based format. Thus the assay was further developed to be able to specifically identify MAP by
combining the phage assay with the IS900 PCR, where the plaques formed on the plates were picked and screened for these MAP-specific DNA sequences. This assay was subsequently used to detect MAP in a range of matrices including, milk, cheese, infant powdered formula and blood. There is great difficulty in assessing the sensitivity and specificity of any new diagnostic test especially in the absence of a gold standard. However Botsaris et al. had demonstrated that using the phage-PCR assay, one could accurately predict with a sensitivity of 90% and specificity of 99% when a bulk tank milk sample was positive for MAP based on the number of plaques. Most recently this assay has been used to detect viable MAP cells in retail milk, where viable MAP cells were detected in 10% of pasteurised milk in England. However this technology is still reliant on classic microbiological methods and techniques, which are not particularly suitable for high-throughput samples.

This phage technology has now advanced to a single tube format (Actiphage®, PBD Biotech), which is more sensitive than the original phage amplification assay and suitable for use on a large number of samples and relies on the four characteristics for phage technology (Table 1). The Actiphage® assay again relies on the use of bacteriophage, however there is no need for plating or incubation with fast growing mycobacteria. This assay essentially uses the phage’s ability to lyse mycobacteria resulting in the efficient release of genomic mycobacterial DNA (Fig. 1). The limitations of the original phage assay, where plaques were picked and scrutinised for signature DNA sequences, are negated using this new technique, as a simple PCR or nucleic acid amplification event is carried out directly on the sample to detect signature MAP DNA sequences. The presence of other mycobacteria or other bacteria does not affect the assay, as specificity of the assay is all predicated on the amplification method used (Fig. 2).

The data generated using the phage assay has demonstrated that viable mycobacteria can be present in the blood of cattle before reaching the latter stages of infection, where viable MAP cells detected in cattle were either inconclusive or negative by milk and serum ELISA in both naturally and experimentally infected cattle. Indeed a recent study in France has demonstrated that MAP can be found in the blood of calves from a Johne’s infected at less than one month of age. However it is
difficult to validate a new test for a disease such as Johne’s, where there is no appropriate gold standard and as such a detection event would be seen as a false positive when compared to the insensitive ELISA or culture, thus raising questions about the specificity of the assay, whereas a detection event can only happen in certain circumstances (Table 1). Here a larger study is needed where further data are required to understand the performance of the phage assay in a wider range of samples. In the absence of a Gold Standard, other statistical approaches would be required such as Bayesian analysis, to begin to predict the performance of phage technology as a diagnostic. These data however, may also provide a tool to delve into the complexities of Johne’s disease to ask novel questions about the pathophysiology of infection, ideally resulting in more information for the control of this disease.

A new tool in the armoury?

Overall, current diagnostic assays for MAP based on serology or faecal testing have poor sensitivities and cannot detect early stages of infection, therefore there is need to find new diagnostic markers for early infection detection and disease stages. DNA amplification technologies, when applied in the right format have the potential to be both specific and sensitive, however processing steps required for efficient DNA amplification are not currently suitable to make the most of the ability of PCR to detect very low levels of DNA. On an individual cow basis, where an ideal approach would be to use serological assays to screen herds for the presence of Johne’s disease, then to use a more sensitive and robust tool that has the specificity of PCR, but with an improved sensitivity, allow detection of Johne’s disease at a much early stage of infection in individual animals, thus giving veterinarians and farmers another tool to control Johne’s disease.

The ability to rapidly detect and specifically identify mycobacteria responsible for infections has been very difficult to achieve due the slow growing, fastidious natures of organisms such a MAP. Phage technology has opened to the door to not only detecting these organisms far quicker than traditional culture, but also allows novel aspects of the pathophysiological nature of infection to be
studied and understood to fully comprehend diseases just as Johne’s disease. Phage technology is an attractive new tool which has the potentially to be used in combination with existing technologies and management schemes to improve and reduce the burden of Johne’s disease on farmers and veterinarians.

Keywords
Johne’s disease; bacteriophage; phage; paratuberculosis; MAP; detection

Key Points
Novel detection methods are needed to control Johne’s disease. Current diagnostics for Johne’s disease are ill-suited for early detection or on an individual animal basis.
Phage technology can rapidly, sensitively and specifically detect *M. paratuberculosis* in blood and milk
Phage technology may be used as another tool in the armoury to tackle Johne’s disease.
References


Fig 1. Bacteriophage lysis

The efficient lysis of bacteria caused by bacteriophage (white arrows). Figure adapted from Roach & Debarbieux, 2017).
Diagram shows the stages of receiving a biological sample, to results. 1. Delivery of sample to the laboratory, 2. Processing at a central facility, 3. Isolation of peripheral blood mononuclear cells where mycobacteria are present, 4. Add phage for mycobacteria to sample, 5. Allow phage to infect any mycobacteria present in the sample, 6. Isolate DNA after cell lysis, 7. Perform DNA amplification of signature MAP DNA.
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<tr>
<th>PHAGE REQUIREMENTS</th>
<th>Description</th>
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<tr>
<td>BINDING</td>
<td>Phage will only bind to specific bacterium. Here phage D29 will ONLY bind to <em>Mycobacterium</em> and no other bacteria</td>
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<tr>
<td>REPLICATION</td>
<td>This will only occur inside a VIABLE host, where phage will hijack their bacterial hosts machinery and replicate</td>
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<td>LYSIS</td>
<td>Specific enzymes are made when the phage is ready to break open their host cell</td>
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<td>DNA RELEASE</td>
<td>This DNA is efficiently released from inside the cell and a species specific PCR is carried out to confirm the identity of the mycobacteria present</td>
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