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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.[9, 16] 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.[29] 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.[24] This long incubation period before Johne's disease becomes detectable using conventional tests makes effective control schemes difficult to implement.

27

28 **Tried and tested: current tools for diagnosing Johne's disease**

29 Although classical laboratory culture of MAP is considered the gold standard method of
30 detection, in practice it is very difficult to perform because MAP is a slow growing organism that is
31 both difficult and expensive to culture. It can take over 16 weeks to culture the bacterium on
32 laboratory media and there are high failure rates due to the high risk of contamination by faster
33 growing organisms.[30] To counter this problem, samples such as milk, faeces or tissue biopsies are
34 decontaminated using chemicals, but these can also affect the viability of the MAP cells in the sample,
35 thus reducing the sensitivity of culture further.[5] Not only does this inability to routinely culture MAP
36 make the gold standard diagnostic test impractical, it also means that fundamental aspects of the
37 pathophysiology of Johne's disease are not well understood, which hinders the development of
38 control programmes.

39 There are a range of immunological-based diagnostics that have been developed to diagnose
40 Johne's disease. There are many commercial and in-house enzyme-linked immunosorbent assays
41 (ELISA) to detect anti-MAP antibodies in bovine milk, serum or plasma. However there can be issues
42 with cross-reactions occurring if the animals have been exposed to other pathogenic or environmental
43 mycobacteria which may result in false-positive results.[23] Another issue identified is that depending
44 on the age and stage of infection, sensitivity of detection can be as low at 2 % in calves to 55 % in older
45 cattle.[11, 12] Poor sensitivity of ElSA tests remains a significant problem and is an unavoidable
46 consequence of the chronic nature of the disease. Since commercial ELISA tests are generally only
47 capable of routinely diagnosing Johne's disease in animals at the later stages of infection, or only
48 during the very late stages of the subclinical phase of infection, they are not really sensitive enough
49 to be used on an individual animal basis to reliably confirm infection. Most control programmes test
50 herds annually,[23] however to compensate for the lack of sensitivity, and the fact that antibody levels
51 in infected animals are known to fluctuate, quarterly testing of individual animals is often carried out,
52 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.[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*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*900*-like sequences have been reported in the literature.[6, 7] The fact that IS*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.[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.[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.[2]

A new approach: Phage Technology

79 Bacteriophage are viruses that infect bacteria and they will only successfully replicate within
80 a viable host. Like all viruses they identify their correct host cells by binding to specific structures
81 (receptors) on the outside of the bacterial cells. Once bound, phage inject their own DNA into the
82 bacterial cell and then take over its machinery to make many copies of themselves before finally
83 producing enzymes that break down the cell wall and cause the cell to break open, releasing new
84 phage particles into the environment (Table 1). Phage have been used for many years to rapidly detect
85 different bacterial pathogens, including members of the *Mycobacterium* genus.[20] Several different
86 phage-based detection assays have been described, such as genetically engineered reporter-phage
87 that produce a fluorescent or bioluminescent signal when they infect their host cells, or phage binding
88 to host cells can be detected using physical methods such as plasmon resonance-based spectrometry
89 (for a full review see Schofield et al., 2012).[20] However more recently the detection of mycobacteria
90 using bacteriophage amplification technology has been developed where the natural life cycle of the
91 phage is exploited to detect its bacterial host.
92 Bacteriophage amplification technology has been exploited to detect a range of mycobacteria.
93 One of the original uses that was commercialised was to detect *M. tuberculosis* -the causative agent
94 of tuberculosis (TB) - in human sputum samples.[1] The assay was a low cost and simple petri-dish based
95 test that enabled mycobacteria to be detected within 48 h (for review see Rees & Botsaris, 2012).[18] To
96 develop this assay a broad spectrum bacteriophage, D29, was used which is capable of detecting a
97 wide range of mycobacteria, including pathogenic and non-pathogenic species. The ability of the assay
98 to detect many strains, led to the phage amplification assay being applied in the agricultural and food
99 sectors. However, the broad host range of phage D29 was both an advantage and disadvantage where
100 in human clinical samples, mycobacteria in the sputum would be treated in the same way regardless
101 of what species was present. However in food and agriculture settings, many other mycobacterial
102 species, both pathogenic and environmental may be present in the samples. This results in the
103 detection of a range of mycobacteria not of interest reducing the specificity of the assay in this plate
104 based format. Thus the assay was further developed to be able to specifically identify MAP by

105 combining the phage assay with the IS*900* PCR, where the plaques formed on the plates were picked
106 and screened for these MAP-specific DNA sequences.[22] This assay was subsequently used to detect
107 MAP in a range of matrices including, milk, cheese, infant powdered formula and blood.[3, 4, 25] There is
108 great difficulty in assessing the sensitivity and specificity of any new diagnostic test especially in the
109 absence of a gold standard. However Botsaris *et al,*[3] had demonstrated that using the phage-PCR
110 assay, one could accurately predict with a sensitivity of 90 % and specificity of 99 % when a bulk tank
111 milk sample was positive for MAP based on the number of plaques. Most recently this assay has been
112 used to detect viable MAP cells in retail milk, where viable MAP cells were detected in 10 % of
113 pasteurised milk in England.[8] However this technology is still reliant on classic microbiological methods
114 and techniques, which are not particularly suitable for high-throughput samples.
115 This phage technology has now advanced to a single tube format (Actiphage®, PBD Biotech),
116 which is more sensitive than the original phage amplification assay[27] and suitable for use on a large
117 number of samples and relies on the four characteristics for phage technology (Table 1). The
118 Actiphage® assay again relies on the use of bacteriophage, however there is no need for plating or
119 incubation with fast growing mycobacteria. This assay essentially uses the phage's ability to lyse
120 mycobacteria resulting in the efficient release of genomic mycobacterial DNA (Fig. 1). The limitations
121 of the original phage assay, where plaques were picked and scrutinised for signature DNA sequences,
122 are negated using this new technique, as a simple PCR or nucleic acid amplification event is carried
123 out directly on the sample to detect signature MAP DNA sequences. The presence of other
124 mycobacteria or other bacteria does not affect the assay, as specificity of the assay is all predicated
125 on the amplification method used (Fig. 2).
126 The data generated using the phage assay has demonstrated that viable mycobacteria can be
127 present in the blood of cattle before reaching the latter stages of infection,[25, 26] where viable MAP cells
128 detected in cattle were either inconclusive or negative by milk and serum ELISA in both naturally and
129 experimentally infected cattle. Indeed a recent study in France has demonstrated that MAP can be
130 found in the blood of calves from a Johne's infected at less than one month of age.[17] However it is

131 difficult to validate a new test for a disease such at Johne's, where there is no appropriate gold
132 standard and as such a detection event would be seen as a false positive when compared to the
133 insensitive ELISA or culture, thus raising questions about the specificity of the assay, whereas a
134 detection event can only happen in certain circumstances (Table 1). Here a larger study is needed
135 where further data are required to understand the performance of the phage assay in a wider range
136 of samples. In the absence of a Gold Standard, other statistical approaches would be required such as
137 Bayesian analysis, to begin to predict the performance of phage technology as a diagnostic.[28] These
138 data however, may also provide a tool to delve into the complexities of Johne's disease to ask novel
139 questions about the pathophysiology of infection, ideally resulting in more information for the control
140 of this disease.
141

142 **A new tool in the armoury?**

143 Overall, current diagnostic assays for MAP based on serology or faecal testing have poor
144 sensitivities and cannot detect early stages of infection, therefore there is need to find new diagnostic
145 markers for early infection detection and disease stages.[13] DNA amplification technologies, when
146 applied in the right format have the potential to be both specific and sensitive, however processing
147 steps required for efficient DNA amplification are not currently suitable to make the most of the ability
148 of PCR to detect very low levels of DNA. On an individual cow basis, where an ideal approach would
149 be to use serological assays to screen herds for the presence of Johne's disease, then to use a more
150 sensitive and robust tool that has the specificity of PCR, but with an improved sensitivity, allow
151 detection of Johne's disease at a much early stage of infection in individual animals, thus giving
152 veterinarians and farmers another tool to control Johne's disease.
153 The ability to rapidly detect and specifically identify mycobacteria responsible for infections
154 has been very difficult to achieve due the slow growing, fastidious natures of organisms such a MAP.
155 Phage technology has opened to the door to not only detecting these organisms far quicker than
156 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* is blood and milk

Phage technology may be used as another tool in the armoury to tackle Johne's disease.

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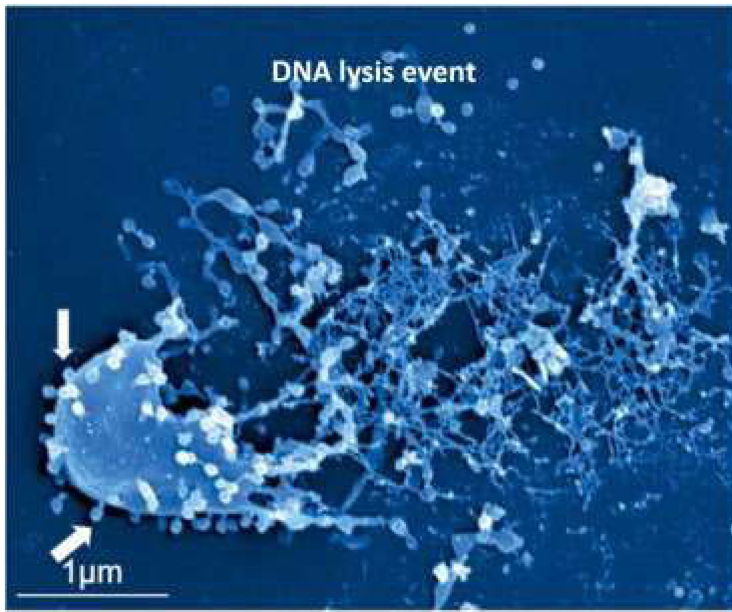
280 **Fig 1. Bacteriophage lysis**

281 

282 The efficient lysis of bacteria caused by bacteriophage (white arrows). Figure adapted from Roach &
283 Debarbieux, 2017).[19]

Fig 2. Schematic of Actiphage® Assay

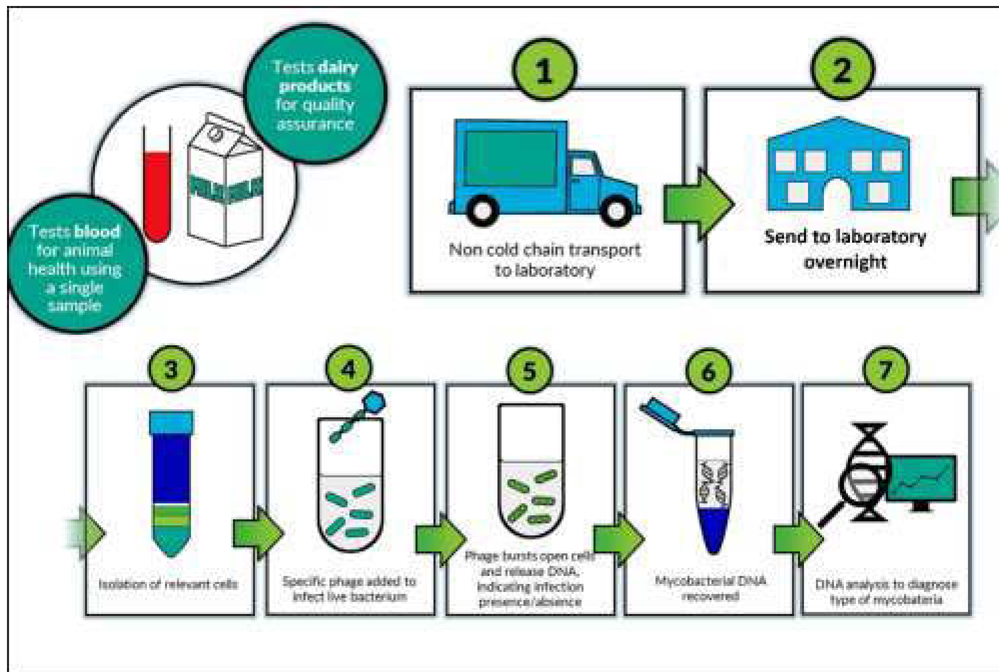


Diagram shows the stages of receiving a biological sample, to results. 1. Delivery of sample to the laboratory, 2. Processing at a central facility, 3. Isolation of peripheral blood mononuclear cells where mycobacteria are present, 4. Add phage for mycobacteria to sample, 5. Allow phage to infect any mycobacteria present in the sample, 6. Isolate DNA after cell lysis, 7. Perform DNA amplification of signature MAP DNA.

295 Table 1. Steps required by phage to enable a successful detection event

PHAGE REQUIREMENTS	
BINDING	Phage will only bind to specific bacterium. Here phage D29 will ONLY bind to *Mycobacterium* and no other bacteria
REPLICATION	This will only occur inside a VIABLE host, where phage will hijack their bacterial hosts machinery and replicate
LYSIS	Specific enzymes are made when the phage is ready to break open their host cell
DNA RELEASE	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

296