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Development of a rapid phage-based method for the detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in blood within 48 h☆

Benjamin M.C. Swift [a,*], Emily J. Denton [b], Sophie A. Mahendran [b], Jonathan N. Huxley [b], Catherine E.D. Rees [a]

[a] School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leics, LE12 5RD, UK
[b] School of Veterinary and Medicine Science, University of Nottingham, Sutton Bonington Campus, Loughborough, Leics, LE12 5RD, UK

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ABSTRACT

The aim of this study was to develop a methodology to rapidly detect viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in clinical blood samples. MAP cells spiked into commercially available blood were recovered using optimised peptide-mediated magnetic separation (PMMS) and detected using a phage-based method, and the identity of the cells detected confirmed using nested-PCR amplification of MAP signature sequences (IS*900*). The limit of detection was determined to be 10 MAP cells per ml of blood and was used to detect MAP present in clinical bovine blood samples. Using the PMMS-phage method there was no difference when detecting MAP from whole blood or from isolated buffy coat. MAP was detected in animals that were milk-ELISA positive (15 animals) by PMMS-phage and no MAP was detected in blood samples from an accredited Johne's disease free herd (5 animals). In a set of samples from one herd (10 animals) that came from animals with variable milk ELISA status, the PMMS-phage results agreed with the positive milk-ELISA results in all but one case. These results show that the PMMS-phage method can detect MAP present in naturally infected blood. Total assay time is 48 h and, unlike PCR-based detection tests, only viable cells are detected. A rapid method for detecting MAP in blood could further the understanding of disseminated infection in animals with Johne's disease.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease which is a wasting disease of cattle and other ruminants that results in lower meat and milk yields, and significant financial losses to both the dairy and beef industries (Raizman et al., 2009). Paratuberculosis occurs throughout the world and is considered endemic in many countries (Fridriksdottir et al., 1999). MAP is transmitted vertically through contaminated milk and colostrum or horizontally via contaminated feed (Whittington and Sergeant, 2001). The disease can be controlled but early detection and reliable diagnostics are paramount in stopping transmission between animals. In Europe herd prevalence ranges from 7% to 55% but the subclinical nature of the disease and the limitations of available diagnostic tests can result in underreporting (Manning and Collins, 2001).

The identification of MAP in the blood of animals susceptible to Johne's disease has been carried out using techniques such as PCR and culture (see Bower et al., 2010 for a review). The use of PCR, although rapid does not distinguish between live and dead organisms. Culture is extremely slow and can take up to 16 weeks to form colonies and in some cases requires decontamination, which can reduce the number of viable cells in a sample (Grant et al., 2003; Gumber and Whittington, 2007). Although Bower et al. (2010) has indicated that some decontamination methods may affect certain MAP strains more than others. Most recently Bower et al. (2010) has described an optimised method for culture of MAP from decontaminated erythrocyte-lysed washed buffy coat, with a reported sensitivity of 10 MAP per ml of spiked whole blood, but this still required up to 12 weeks incubation. Hence this long time required for culture can hamper efforts to understand different phases of disease and determining when an animal develops disseminated infection.

The *FASTplaqueTB™* assay (FPTB; Lab21, UK) is a phage-based detection method for human tuberculosis that has been adapted for the detection of viable MAP in milk and cheese (Altic et al., 2007; Botsaris et al., 2010; Stanley et al., 2007). To increase the specificity of the method the phage-based assay was combined with PCR amplification of the multi-copy IS*900* element. During the development of the phage-based assay it was noted that substances present in the sample matrix can inhibit phage infection. Sample processing was required to ensure these are removed. Magnetic separation is a very simple method of capturing and concentrating cells from a matrix using magnet beads coated with a specific binding agent (either antibody

Abbreviations: PMMS, Peptide mediated magnetic separation; FPTB, *FASTplaqueTB* assay; MP, Media Plus.

* Corresponding author. Tel.: +44 1159516161.
E-mail address: stxbs@nottingham.ac.uk (B.M.C. Swift).

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or peptide). MAP-specific binding peptides coupled to magnetic beads described by Stratmann et al. (2006), have been used to recover MAP cells from milk samples (Foddai et al., 2010).

The aim of this study was to combine existing technologies to develop a novel protocol that could be used to rapidly detect MAP cells in blood using a combined phage-PCR approach, and then to test whether this could be used to detect viable MAP in clinical blood samples.

2. Materials and methods

2.1. Bacterial strains, bacteriophage and growth media

MAP strains K10 and ATCC 19698 were used in the initial experiments to optimise the phage assay. The *Mycobacterium smegmatis* strain and the bacteriophage used (Actiphage) were those supplied in the *FASTPlaqueTB™* kit. All cultures of MAP were prepared using *FASTPlaqueTB™* Media Plus (modified 7H9/OADC media) supplemented with Mycobactin J (2 μg μl^{-1}; Synbiotics Corporation, France).

To culture from beads, 100 μl of each sample, after PMMS (Section 2.3) were inoculated on Herrold's egg yolk medium (HEYM) slopes supplemented with Mycobactin J (BD, France). After inoculation, tubes were incubated slanted with the caps lightly screwed at 37 °C. After one week of inoculation slopes were examined for growth of contaminating organisms. After two weeks the caps were sealed and the slopes incubated up right. The slopes were then examined every week for 16 weeks, and every month up to six months after for growth. After six months, if there was no visible growth, a slope wash detection method was carried out.

2.2. Slope wash detection of MAP growth

The slope wash was based on a method by Williams and Monif (2009). Briefly, 1 ml of sterile reverse osmosis (RO) water was added to a slope with no visible growth. Using a sterile loop, the surface of the agar was gently scraped to loosen any cells present. The slopes were vortexed for 30 s and the liquid transferred to 1.5 ml centrifuge tube and centrifuged (15 min, 17000 ×*g*). The supernatant was removed, frozen at −80 °C and then rapidly thawed before boiling for 10 min to induce cell lysis. The samples were then centrifuged for 3 min (17000 ×*g*) and the supernatant (10 μl) used as template DNA for PCR amplification of IS*900* (Section 2.6).

2.3. Preparation of peptide-coated magnetic beads

Biotinylated peptides were supplied by Cambridge Peptides Ltd. Magnetic beads (Dynabeads - MyOne Tosylactivated, Invitrogen) were individually coated with peptides aMp3 and aMptD (Stratmann et al., 2006) according to the manufacturers' instructions.

2.4. Sample preparation and recovery of MAP cells from blood

For development of the assay, MAP cells were added to commercial horse or sheep's blood (Oxoid, UK). To recover MAP cells, 1 ml blood samples were diluted with 9 ml of Media Plus. Peptide mediated magnetic separation was performed using an adaptation of the method described (Foddai et al., 2010) by adding 10 μl of peptide coated beads (5 μl each of aMp3- and aMptD-coated beads) to each sample and mixing at 18 rpm for 30 min (Dynabeads-MX mixer, Invitrogen). Beads and bound MAP cells were recovered by centrifugation at 4500 ×*g* for 15 min. The supernatant was removed and the beads washed using 9 ml of fresh Media Plus. The beads were again recovered by centrifugation (4500 ×*g*, 15 min) and finally resuspended in 1 ml of Media Plus before being transferred into a microcentrifuge tube for PMMS. Finally, the beads resuspended in 1 ml of Media Plus and Mycobacteria cells present detected using the FPTB assay reagents.

2.5. Detection and enumeration of MAP using the FASTPlaqueTB™ (FPTB) assay

The FPTB (Lab21, Cambridge, UK) assay was carried out according to the manufacturer's instructions. To perform the assay, samples containing MAP are mixed with a broad spectrum mycobacteriophage; D29. After the infection period any extracellular phage are inactivated using a virucide; only phage that have successfully infected a cell are protected from the virucide. The virucide is then neutralised by dilution and infected cells are then plated in a lawn of fast growing *M. smegmatis* using soft agar. Lysis of the infected cells releases new phage which then infect the *M. smegmatis* cells and leads to the formation of a plaque in the lawn. Hence each plaque formed represents one MAP cell in the original sample. Standard FPTB assay positive and negative controls were used each time the assay was performed. Enumeration of MAP cells in inocula was determined using the modification of the FPTB assay as described by Rees and Botsaris (2012) which involves diluting samples until countable numbers of plaques are obtained (data reported as pfu ml^{-1}).

2.6. PCR for the identification of MAP cells

When a MAP cell is present in the initial sample, its DNA is preserved in the centre of the plaque (Stanley et al., 2007). Identification of the cell detected within single plaques was achieved by PCR amplification of IS*900* signature sequences from this DNA using a modification of the plaque-PCR method described by (Botsaris et al., 2010). In this study DNA was extracted from five plaques and concentrated from plaque agar using Zymo-Gel DNA Recovery Spin Columns™, (ZymoResearch, USA). IS*900*-specific nested PCR (Bull et al., 2003) was performed using a 5 μl sample of DNA as template. Purified MAP K10 DNA was used as a positive PCR control and DNA extracted from plaques only containing *M. smegmatis* cells (the FPTB positive control samples) as a negative control.

2.7. Detection of MAP in clinical blood samples

Blood samples were provided as superfluous material under the Veterinary Surgeons Act as part of an on-going herd health screening programme. The study protocol was approved by the University of Nottingham, School of Veterinary Medicine and Science ethical review panel prior to sample usage. Blood samples were collected from nine cows, which had produced positive Johne's milk ELISA test results on three separate occasions (Set A). Blood samples were collected from five cows that belong to an accredited Johne's disease-free herd (Set B). Before sampling, the site of venipuncture was cleaned twice with alcohol. Blood was drawn into either sterile sodium heparin Vacutainer tubes (for phage assay) or plain Vacutainer tubes for blood ELISA. The blood ELISA was performed by a commercial laboratory (Nationwide Laboratories, Leeds, UK).

2.8. Isolation of buffy coat and plasma

The isolation of the buffy coat from cows blood was carried out using Ficoll-Paque Plus (GE Healthcare Life Sciences, UK). The buffy coat layer from 2 ml of whole blood was isolated according to the manufacturer's instructions. The plasma layer was also taken and resuspended after the final centrifugation in MP. The samples were then processed as whole blood in Section 2.4.

3. Results

3.1. Optimisation of PMMS and sample preparation

To develop the method, MAP cells were spiked into commercially available blood. To determine the efficiency of the PMMS recovery, 3.5×10^1 pfu.ml^{-1} was spiked into blood. Magnetic recovery of