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Sensitive and specific detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in raw milk by the Peptide-mediated magnetic separation (PMS)-phage assay

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ABSTRACT

Aim: To validate an optimised Peptide-mediated magnetic separation (PMS)-Phage assay for detection of viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in milk.

Methods and Results: Inclusivity, specificity and limit of detection 50% (LOD_{50}) of the optimised PMS-phage assay were assessed. Plaques were obtained for all 43 MAP strains tested. Of 12 other *Mycobacterium* spp. tested, only *M. bovis* BCG produced small numbers of plaques. LOD_{50} of the PMS-phage assay was 0.93 MAP cells per 50 ml milk, which was better than both PMS-qPCR and PMS-culture. When individual milks (n=146) and bulk tank milk (BTM, n=22) obtained from Johne's affected herds were tested by the PMS-phage assay, viable MAP were detected in 31 (21.2%) of 146 individual milks and 13 (59.1%) of 22 BTM, with MAP numbers detected ranging from 6-948 PFU per 50 ml milk. PMS-qPCR and PMS-MGIT culture proved to be less sensitive tests than the PMS-phage assay.

Conclusions: The optimised PMS-phage assay is the most sensitive and specific method available for the detection of viable MAP in milk. Further work is needed to streamline the PMS-phage assay, because the assay's multi-step format currently makes it unsuitable for adoption by the dairy industry as a screening test.

Significance and Impact of the study: The inclusivity (ability to detect all MAP strains), specificity (ability to detect only MAP), and detection sensitivity (ability to detect low numbers of MAP) of the optimised PMS-phage assay have been comprehensively demonstrated for the first time.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), PMS-phage assay, detection sensitivity, detection specificity, milk testing

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic enteric wasting disease primarily affecting domestic ruminants (Stabel 1998; Sweeney 2011). Infected animals shed the pathogen in their faeces and milk. JD is found throughout the world and many different estimates of herd prevalence have been reported (Manning and Collins 2001; Tiwari *et al.* 2006; Wilson *et al.* 2010). However, the true prevalence of JD among farmed animals is unknown and, because of limitations of current methodologies for the detection of MAP, levels of MAP infection reported are likely to be underestimates in many cases. A study carried out by Nielsen and Toft (2009) reviewed many prevalence studies carried out across Europe and concluded that the prevalence of JD is likely to be >50% in many European countries.

There is some evidence to suggest an association between MAP and various chronic long-term human disorders, including Crohn's disease (CD), irritable bowel syndrome, Type 1 diabetes (T1DM), Multiple Sclerosis (MS), and more recently HIV infection, sarcoidosis, and Hashimoto Thyroiditis (Waddel *et al.* 2015). Although the role of MAP in the development, or progression, of any of these human diseases is still unclear, recent meta-analysis studies demonstrated a significant association with at least three of these human disorders including CD (Feller *et al.* 2007; Waddel *et al.* 2015; Timms *et al.* 2016), T1DM and MS (Waddel *et al.* 2015). Uncertainty about the role of MAP as a human pathogen still remains, and there is general consensus within the food safety community that human exposure to MAP should be minimized as a precautionary measure. Consumption of milk, and possibly meat, from infected animals is currently viewed as a potential source of zoonotic transmission of the

pathogen from animals to humans. MAP has been isolated from retail milk and dairy products in many parts of the world (Grant *et al.* 2002; Ayele *et al.* 2005; Ellingson *et al.* 2005; Carvalho *et al.* 2012; Paolicchi *et al.* 2012) suggesting that the pathogen can enter the human food chain and that current High Temperature, Short Time (HTST) pasteurization may not always ensure complete inactivation of this pathogenic bacterium.

Traditional cultural methods cannot be routinely adopted to demonstrate the presence of viable MAP in milk because the methods are time-consuming, not specific and lack sensitivity (Slana *et al.* 2008a). Indeed, chemical decontamination applied before culture to inactivate the competitive flora has been proven to have a detrimental effect on the viability of MAP (Grant *et al.* 2003; Gao *et al.* 2005), and also to extend the time required for primary isolation. A number of molecular tests including both conventional and quantitative PCR methods have been successfully developed to permit faster detection of MAP in milk (Timms *et al.* 2011). However, most of these tests do not provide information about the viability of detected cells and their sensitivity is generally affected by non-mycobacterial DNA, protein and PCR inhibitors present in test samples, unless laborious DNA extraction methods are applied before DNA amplification (Timms *et al.* 2015).

We recently combined an optimized phage amplification assay (Foddai *et al.* 2009) with selective peptide-mediated magnetic separation (PMS) (Foddai *et al.* 2010b) to achieve a rapid novel detection test for viable MAP. The PMS-phage method exploits the use of D29 mycobacteriophage and is able to provide rapid enumeration of viable MAP in milk and veterinary samples within 48 h (Foddai *et al.* 2011), based on the number of plaques (lysis areas) produced on a lawn of fast-growing *M. smegmatis*. More recently, an optimized milk sample preparation protocol

to maximize accuracy of MAP counts when testing cows' milk was described (Foddai and Grant 2015). The aims of the present study were: (1) to assess inclusivity and specificity of the recently optimised PMS-phage assay by testing a large number of MAP strains and other bacterial milk isolates; (2) to compare the limit of detection 50% (LOD_{50}) of the optimised PMS-phage assay with those of PMS-culture and PMS-qPCR targeting both IS900 and f57 by testing artificially contaminated milk samples; and (3) to test individual raw milk samples and bulk tank milk from dairy herds affected by JD to assess the applicability of the optimised PMS-phage assay for raw milk testing.

Materials and methods

Bacterial strains and growth conditions

Forty-three MAP strains (three type strains and 40 cattle, raw and pasteurised cow's milk, untreated water, or Crohn's disease isolates, acquired or isolated over the past 20 years and available within the Queen's University Belfast (QUB) culture collection), 12 other *Mycobacterium* spp. (Table 1), and five bacterial isolates (one Gram positive coccus and four Gram positive rods) obtained by plating a sample of raw cows' milk on Nutrient agar (Oxoid Ltd., Basingstoke, UK), were used in this study. All MAP strains were grown in a shaker incubator for 4-6 weeks at 37ºC to stationary phase in Middlebrook 7H9 broth containing 10% (v/v) OADC supplement (both from Difco) and 2 µg per ml mycobactin J (Synbiotics Europe SAS, Lyon, France). All *Mycobacterium* spp. were cultivated at appropriate temperatures (dependent on species) to stationary phase (between 3 and 15 days) in the same

7H9 medium without the addition of mycobactin J. Milk isolates were grown overnight in 10 ml of nutrient broth at 25°C.

Peptide-mediated magnetic separation (PMS)

PMS was performed on 1 ml of sample using 5 µL biotinylated-aMp3 peptide- and 5 µL biotinylated-aMptD peptide-coated MyOne™ Tosylactivated Dynabeads® (Life Technologies), prepared in-house as previously described (Foddai *et al.* 2010b). Magnetic separation was carried out using the Dynal BeadRetriever (Life Technologies). Magnetic capture was carried out for 30 min at room temperature under continuous mixing, followed by two washes in 1 ml Phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBS-T20, Sigma), and final resuspension of the beads in 1 ml 7H9 broth containing 10% (v/v) OADC.

Optimised phage amplification assay

The optimised phage assay was carried out as previously described by Foddai *et al.* (2009). Briefly, after overnight incubation of samples at 37ºC in 1 ml of 7H9 medium containing 2 mmol l^{-1} $CaCl_2$, samples were incubated for 2 hours at 37ºC with 10^8 D29 mycobacteriophage before treatment with 100 mmol l^{-1} ferrous ammonium sulphate (FAS, Sigma) for 10 min at room temperature to inactivate any exogenous/non-adsorbed seed phage. Samples were then mixed with 5 ml 7H9 medium containing 2 mmol l^{-1} $CaCl_2$ and returned to the incubator at 37ºC for a further 90 min before being plated with tempered 7H9 agar and 1 ml *Mycobacterium smegmatis* mc^2 155 (10^8 CFU per ml). Plaques were counted following overnight incubation of plates at 37ºC.

Confirmation of inclusivity of the novel PMS-phage assay by testing a broad range of MAP strains

Before being tested by the optimised PMS-phage assay, all stationary MAP broth cultures were declumped by ultrasonication applied as previously described (Foddai and Grant 2015) at 37 kHz for 4 min on ice in a Ultrasonic PH 30 (Fisher Scientific Ltd) and then tested for purity (presence of only red acid-fast cells) by Ziehl–Neelsen (ZN) staining. The number of cells per ml of broth was estimated by measuring the optical density at 600nm (OD_{600}) using a WPA CO8000 cell density meter (SISLAB, Italy). For each sample, optical density was adjusted to an OD_{600} of 0.1 (approximately 10^6-10^7 MAP cells per ml) followed by serial dilution of cultures in PBS-T20. PBS-T20 suspensions containing approximately 10^2-10^3 MAP per ml were finally processed through optimised PMS-phage assay to assess inclusivity of the assay. The number of MAP cells detected was indicated by plaques (zones of clearing) produced on agar plates containing 5 ml of molten 7H9 agar and 1 ml of *M. smegmatis* mc^2 155, and reported as plaque-forming-units (PFU) per ml. The experiment was repeated twice for each of the 43 MAP strains.

Confirmation of specificity of the D29-based phage assay by testing various environmental *Mycobacterium* spp. and non-mycobacterial raw milk isolates

Broth suspensions of 12 environmental *Mycobacterium* spp. and five raw milk isolates spiked at three levels (10-10^2, 10^2-10^3, 10^3-10^4 cells per ml) were processed through the phage assay to assess specificity of the assay for MAP. For each sample tested, inoculum was prepared as described above. Number of bacterial cells per ml stationary phase broth was estimated by measuring and adjusting the

original optical density followed by serial dilution of samples in 1 ml PBS-T20. The experiment was repeated twice for each *Mycobacterium* sp. and raw milk isolate.

Comparison of the limit of detection 50% (LOD_{50}) of the PMS-phage assay, PMS-culture and PMS-qPCR

Ultra-high temperature (UHT) milk purchased from a local supermarket and artificially contaminated at different levels with MAP was used to compare the LOD_{50} of the three detection methods. Three sets of 50 ml UHT milk samples were spiked in triplicate at four levels of MAP contamination (target final concentrations 10^2-10^3, 10-10^2, 1-10 and 0 PFU per 50 ml) by adding 1 ml of an appropriate dilution of MAP NCTC 8578 or ATCC 19698 per 50 ml of milk. The number of MAP added at the highest spiking level, in each case, was determined by the optimised phage amplification assay (no PMS) applied to the diluted MAP culture used as inoculum. Each set of 50 ml milk samples was processed through PMS followed by either the phage amplification assay, culture or qPCR. Irrespective of detection method, milk sample preparation included: (i) centrifugation at 2,500 x g for 15 min, (ii) declumping by ultrasonication applied to the resuspended pellet fraction (Foddai and Grant, 2015), (iii) PMS, and then one of the following detection methods:

a) Phage assay applied after a previous overnight incubation of bead samples in 7H9 Middlebrook broth supplemented with 10% (v/v) OADC, 2 mmol l^{-1} $CaCl_2$ and NOA Antimicrobial Supplement (Product code 3A201N-300, Abtek Biological Ltd, Liverpool UK; final concentrations per ml of broth: Nystatin 50 IU, Oxacillin 2 µg, Aztreonam 30 µg);

b) Culture on plates of Herrold's egg yolk medium (HEYM) supplemented with 2 µg per ml mycobactin J and PANTA (Becton Dickinson; final concentrations per ml of

HEYM: 10 IU Polymyxin B, 1 µg Amphotericin, 4 µg Nalidixic acid, 1 µg Trimethoprim and 1 µg Azlocillin);

c) qPCR targeting both IS900 and f57 (Donaghy *et al.* 2010) applied on MAP DNA released from captured cells through heating samples at 95°C for 25 min. All qPCR reactions were performed on an Eco™ Real-Time PCR system (Illumina, Inc).

The limit of detection experiment was carried out with two MAP type strains, ATCC 19698 and NCTC 8578.

Testing of individual and bulk tank milks from Johne's affected herds

A total of 146 individual raw milk samples and three bulk tank milks (BTM) sourced from a large JD affected dairy herd in southern England, identified with the help of Mr Peter Orpin (Park Veterinary Group, Leicester), and 19 BTM obtained from dairy herds in Scotland with the help of Dr George Caldow (SAC Consulting Services, St Boswells) were tested for MAP. The individual milk samples were collected by farm workers during one morning milking session into sterile 50 ml centrifuge tubes supplied by QUB. Milk samples were immediately frozen and stored overnight on farm before being transported to QUB in insulated boxes by an overnight courier. Samples arrived at QUB in a partially frozen state and were immediately placed in -80ºC freezer. Before application of the PMS-phage assay sample preparation included: thawing overnight in the refrigerator at 4ºC, room temperature for 1 h before centrifugation of 50 ml milk at 2500 x g for 15 min at room temperature, resuspension of milk pellet fraction in 1 ml PBS-T20, application of ultrasonication to disperse MAP clumps (Foddai and Grant 2015), and then PMS. After PMS, samples were concentrated in a final volume of 150 µl of sterile RNAse/DNAse free water (Sigma) and divided into three aliquots (50 µl) to be processed via the three different

detection methods - phage amplification assay, culture, and real time qPCR as described below.

Phage amplification assay and confirmatory Plaque PCR

Following overnight incubation of the bead samples at 37ºC in a final volume of 1 ml 7H9 medium supplemented with 10% (v/v) OADC, 2 mmol l^{-1} $CaCl_2$ and NOA Antimicrobial Supplement (as described above), the phage amplification assay was carried out as described by Foddai *et al.* (2009). After overnight incubation, plaques (1 to, maximum, 10 depending on PFU counts) from positive phage assay plates were harvested and processed through DNA extraction as described by Swift *et al.* (2013). Plaque DNA in each aliquot was concentrated and purified through Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, Ca, USA), and then subjected to IS900 Plaque-PCR as described by Stanley *et al.* (2007).

Culture and confirmation of MAP isolation

After PMS, samples were cultivated in BD BBL MGIT™ Mycobacteria Growth Indicator Tubes containing 4 ml of modified Middlebrook liquid broth enriched with 0.5 ml BBL™ MGIT™ OADC and 0.1 ml MGIT™ PANTA™ antibiotic mixture (all Becton Dickinson Limited, USA). Cultures were incubated for 16-20 weeks at 37ºC and were periodically monitored using a BACTEC MicroMGIT reader (Becton Dickinson Limited, USA). Liquid broths showing evidence of growth were tested by ZN staining for the presence acid-fast bacilli and by IS900 PCR (Naser *et al.* 2013) to identify MAP positive cultures. All cultures that tested acid-fast and IS900 PCR positive were then subjected to a mild chemical decontamination treatment in 0.75% (w/v) hexadecylpyridinium chloride (HPC) for 60 min at room temperature before

being sub-cultured onto Herrold's egg yolk agar (HEYM) slopes supplemented with mycobactin J and PANTA. Slopes were incubated at 37°C for over 10 months to achieve isolation of typical colonies and final identification as MAP.

Real time qPCR

Each sample analysed by real time qPCR targeting IS900 and f57 MAP sequences (Donaghy *et al.* 2010) was tested in duplicate. DNA was released from PMS samples by heating samples at 95°C for 25 min. After brief centrifugation at 10,000 g for 1 min to sediment beads, an aliquot of 2.5 µl of the supernatant was used per qPCR reaction. Each qPCR reaction was performed in a final volume of 25 µl including: TaqMan Universal 2X PCR master mix (Applied Biosystems), EXO IPC 10x mix and EXO IPC 10x DNA (Applied Biosystems), 10 µmol l^{-1} of each forward and reverse primer, 5 µmol l^{-1} of specific probe (Donaghy *et al.* 2010), and 2.5 µl template DNA. PCR was performed using an Eco™ Real-Time PCR system (Illumina, Inc) with the following thermal cycling conditions: 50°C for 2 min; 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistical analysis of results

For inclusivity and specificity testing, positive or negative results for the plaque assay applied after PMS were used as a final result. Limit of detection (LOD_{50}) and associated 95% confidence limits of the three methods assays (PMS-phage assay, PMS-culture, PMS-IS900 and f57 qPCR) were estimated using the generalized Spearman-Karber LOD_{50} calculation for 4-level spiking protocols (AOAC International 2006). Kappa agreement between test results was determined using EpiTools Epidemiological Calculators (http://epitools.ausvet.com.au).

Results

Confirmation of inclusivity of the novel PMS-phage assay

All 43 MAP strains tested by the PMS-phage assay yielded plaques, confirming 100% inclusivity of the test (Figure 1). PFU counts observed for most of the MAP strains (40 of 43, 93%) ranged from 100 to 300 (mean 142.26±51.97 PFU per ml); the number of plaques expected based on the culture dilution tested. Three MAP strains (NCTC 8578, 307R and Van Veen 52991-1) showed greater variability in counts between replicates and a higher mean PFU ml^{-1} count (mean 681.50±165.91 PFU per ml) than was observed for the other strains tested (Figure 1).

Confirmation of specificityof the PMS-phage assay

Eleven of the 12 non-target *Mycobacterium* spp. tested negative by the PMS-phage assay (i.e. no plaques observed), as did all five raw milk bacterial isolates tested. Some plaques were observed for *M. bovis* BCG at the highest spiking level (10^3-10^4 CFU per ml) tested only, however the number of plaques (6.5 and 10.5 PFU per ml) after PMS was substantially lower than the original spiked population (data not shown). The mean percentage non-specific recovery of *M. bovis* BCG observed in two separate experiments was 0.19±0.08%.

Comparison of the LOD_{50} of the PMS-phage assay, PMS-culture and PMS-qPCR

Before testing by the three methods, spiked UHT milk samples contained 920, 92 and 9 PFU per 50 ml of MAP ATCC 19698, and 860, 86 and 8 PFU per 50 ml milk of

MAP NCTC 8578; determined by the optimised phage amplification assay (without PMS). Higher detection sensitivity for MAP was exhibited by the PMS-phage assay compared to the PMS-qPCR and PMS-culture assays applied in parallel to spiked milk samples. The LOD_{50} of the PMS-phage assay was estimated to be 0.90 and 0.95 PFU per 50 ml milk (calculation of 95% CI not possible) for MAP strains NCTC 8578 and ATCC 19698, respectively (Table 2). PMS-IS900 qPCR had the next highest detection sensitivity, followed by PMS-f57 qPCR, and finally PMS- culture. The LOD_{50} of PMS-IS900 qPCR was 136.7 (95% CI: 21.4-872.9) and 134.55 (95% CI: 23.45-772.80) MAP cells per 50 ml milk for strains NCTC 8578 and ATCC 19698, respectively; the LOD_{50} of f57 qPCR was 303.7 and 291.00 MAP cells per 50 ml milk (calculaton of 95% CI not possible) for strains NCTC 8578 and MAP ATCC19698, respectively (Table 2). An LOD_{50} for PMS-culture could not be calculated because none of the milk samples spiked at the highest level (10^2-10^3 CFU per 50 ml) yielded colonies on HEYM agar plates, and thus use of the Excel LOD_{50} calculator (AOAC International, 2006) was not valid.

Testing of individual and bulk tank milks from Johne's affected herds

Table 3 summarises the number and percentage of individual and bulk tank milk samples testing MAP positive by the three detection methods. Overall, 59 milk samples yielded plaques in the PMS-phage assay, some or all of which (depending on numbers present) were harvested and subjected to IS900 Plaque-PCR. Of the 59 plaque composites tested by IS900 Plaque-PCR, 44 (31 from individual milks and 13 from BTM, 74.6%) yielded clear, positive IS900 Plaque-PCR results, 5 (8.5%) yielded inconclusive PCR results, 10 (16.9%) yielded negative PCR results, and one was not tested because plaques had merged and individual plaques could not be

harvested. Therefore, viable MAP were confirmed to be present in 31 (21.2 %) of 146 individual milks and 13 (59.1%) of 22 BTM tested by the PMS-phage assay (Table 3). Mean viable MAP counts indicated by the PMS-phage assay in the positive individual raw milks and bulk tank milks were 228.1 PFU per 50 ml (range 6-948 PFU per 50 ml) and 136.83 PFU per 50 ml (range 18-685 PFU per 50 ml), respectively.

Due to budget restrictions, and the relatively high cost of qPCR testing, real time qPCR was only used to test all 22 bulk tank milks and selected individual raw milk samples (n=77, 47 samples that had tested PMS-phage assay positive and 30 other randomly selected samples that had tested PMS-phage assay negative). This may have biased the outcome of the PMS-qPCR tests, so direct comparisons with the outcomes of the PMS-phage assay and PMS-culture will not be made. MAP was detected by qPCR in 7 (9.1%) out of 77 individual raw milk samples and 10 (45.4%) out of 22 BTM tested. IS900 qPCR showed higher detection rates than f57 qPCR, as expected. Of the seven individual raw milks that tested qPCR positive, all 7 (9.1%) tested IS900 qPCR positive and 2 samples (2.6%) tested both IS900 and f57 qPCR positive. Of the 10 BTM that tested qPCR positive, 10 (45.4%) tested IS900 qPCR positive, only 2 (9.1%) tested both IS900 and f57 qPCR positive.

When cultured in MGIT broth (not HEYM agar plates, as in first part of the study) after PMS, 49 (33.6%) of the 146 individual raw milk cultures and 16 (72.7%) of the 22 BTM cultures showed evidence of acid-fast bacteria when tested by ZN staining; many of these cultures were mixed cultures and showed evidence of the presence of non-acid-fast bacteria. Isolation of MAP cells was confirmed in 17 (11.6%) individual raw milk and 11 (50%) BTM cultures by IS900 PCR (Naser *et al.* 2013). Inter-relationships between results of the PMS-phage assay, PMS-culture

and PMS-qPCR applied to individual raw milks and BTM are shown as Venn diagrams in Figure 2. It should be noted in the case of the individual raw milks, 9 of the 17 milk samples that tested PMS-culture positive (Figure 2A) and 2 of the 7 milk samples that tested PMS-qPCR positive (Figure 2B) also yielded plaques with the PMS-phage assay; however the presence of MAP DNA in the plaques harvested was either not confirmed by Plaque-PCR or the plaque-PCR result was inconclusive, and so a PMS-phage assay negative result was recorded. Two BTM samples tested PMS-phage assay negative but PMS-culture, and PMS-culture and qPCR positive (Figure 2C). It is possible that overgrowth of environmental mycobacteria adhering to the peptide-coated magnetic beads going into the phage assay may have masked plaques in the *M. smegmatis* lawns for these samples. As no plaques were observed, these two samples were recorded as PMS-phage assay negative as a result, when potentially this was a false negative result.

Overall, agreement between results of the PMS-phage assay and those of PMS-culture and PMS-qPCR tests was 'fair ' and "poor", respectively, when 146 individual milks were tested (PMS-phage v PMS-culture: Kappa 0.25, 95% CI: 0.062 to 0.439, *P*=0.0028; PMS-phage v PMS-IS900 qPCR: Kappa 0.073, 95% CI: -0.084 to 0.231, *P*=0.1695), whereas it was 'moderate' in both cases when 22 BTMs were tested (PMS-phage v PMS-culture: Kappa 0.538, 95% CI: 0.184 to 0.892, *P*=0.0056; PMS-phage v PMS-IS900 qPCR: Kappa 0.553, 95% CI: 0.220 to 0.885, *P*=0.0036).

Discussion

Culture is still generally considered the 'gold standard' method to demonstrate the presence of viable MAP in test samples. However, the method is time consuming, takes weeks to yield results, and any MAP counts obtained are going to be

underestimates due to the inclusion of a chemical decontamination step prior to culture which can adversely impact MAP viability. There is interest amongst dairy producers and processors to identify a rapid method that could be adopted to detect the presence of MAP in raw milk from primary suppliers and dairy products produced from this milk. The aim of this study was to evaluate the performance of an already described PMS-phage assay (Foddai *et al.* 2011) for the rapid detection of viable MAP in cows' milk that has undergone further optimisation over recent years (Foddai and Grant, 2015). The method exploits the ability of D29 mycobacteriophage to replicate and amplify within only viable mycobacterial cells and represents an optimized version (Foddai *et al.* 2009; Foddai *et al.* 2010a) of the commercially phage-based test (*FASTPlaque*TB™ assay, Biotec Laboratories Limited, Ipswich), which was originally developed for the rapid detection of viable *M. tuberculosis*. In order to maximize specificity of detection, the phage amplification assay is applied after selective capture of MAP cells on paramagnetic beads coated with two selective anti-MAP peptide binders (Foddai *et al.* 2010b). To date, only results of PMS-phage assay testing of a limited number of naturally contaminated BTM (n=44) and faeces (n=39) samples have been reported, providing proof-of-concept for the PMS-phage assay (Foddai *et al.* 2011). Further refinements to the milk testing procedure, mostly related to optimising milk sample preparation prior to PMS, have been made since 2011. Therefore, this study was carried out to assess inclusivity, specificity and detection sensitivity of an optimised version of the PMS-phage assay, and to compare this assay with PMS-culture and PMS-qPCR.

Inclusivity of a microbiological test is the ability to detect the target microorganism within a wide range of bacterial strains. Prior to this study a limited range of MAP strains had been tested by PMS and also by the phage amplification

assay (Foddai *et al.* 2009; Foddai *et al.* 2010a, 2010b). A much broader range of MAP strains (n= 43) isolated from various sources (animals, milk, water and humans) was tested as part of this study to demonstrate inclusivity for MAP of the two peptide binders aMp3 and aMptD involved in the PMS, and of the D29 mycobacteriophage involved in the phage amplification assay. The observation of plaques when the MAP strains were tested demonstrated that all strains were successfully infected by D29 mycobacteriophage, and this finding confirmed 100% inclusivity for MAP. Although some variability in PFU counts was observed, similar plaque counts were observed for the vast majority (93%) of MAP strains tested. This also suggests that consistent capture was being achieved by the two peptide binders during PMS. Due to the hydrophobic nature of its cell wall, MAP cells tend to aggregate in clumps, the presence of which impacts accurate enumeration of viable MAP cells in tested samples. The appearance of the MAP broths before testing visibly differed between strains, likely due to variations in clump size and distribution, and even after de-clumping some broths still showed visible clumps that had not been fully dispersed. These observations might explain the variability in PFU counts observed for different MAP cultures, and lower PFU counts may have resulted due to counting of clumps of cells rather than just single cells. Conversely, MAP cultures that contained larger clumps that were effectively dispersed into single cells by ultrasonication treatment would yield higher PFU counts; which was apparently the case for MAP strains NCTC 8578, 307R and Van Veen 52991-1. In the case of MAP strains showing particularly large standard deviations in Figure 1, the efficiency of the de-clumping step has clearly differed more between the duplicate MAP suspensions tested for these strains compared to the other MAP strains tested. The latter finding suggests that different degrees of de-clumping were being achieved by

the ultrasonication treatment applied before PMS; thus ultrasonication conditions for MAP de-clumping purposes may need further optimization.

In relation to specificity, this study assessed if the D29 mycobacteriophage involved in the phage assay was capable of infecting other *Mycobacterium* spp., or other raw milk bacteria, which might potentially lead to formation of plaques in the absence of MAP when the method is used to test milk samples. A previous study carried out by Rybniker *et al.* (2006) reported that the D29 mycobacteriophage can infect various *Mycobacterium* spp. including *M. smegmatis*, *M. tuberculosis*, *M. bovis* BCG, *M. avium*, *M. scrofulaceum* and *M. ulcerans,* whereas it was unable to infect *M. marinum*, *M. fortuitum* and *M. chelonae*. The assay used in this study was modified for MAP based on its specific burst time, which was found to be longer than for other *Mycobacterium* spp. (Foddai *et al.* 2009). As cell lysis is dependent on the host's generation time, the D29 phage requires a longer time (220 min) within MAP cells to replicate and release the new phage progeny than in other faster-growing *Mycobacterium* spp.; the burst time of which generally ranges between 1 and 2 hours (David *et al.* 1980). Consequently, the virucidal treatment applied after two hours of incubation with D29 bacteriophage would have no detrimental effect against phage particles still to be released from MAP cells, whereas it inactivates any progeny phage already released from faster-growing mycobacteria. This explains the absence of plaques for almost all *Mycobacterium* spp. tested in this study. Some plaques were only observed with *M. bovis* BCG, which is the sole *Mycobacterium* sp. tested with a similar burst time to MAP (180 min; Foddai and Grant unpublished data). However, minimal recovery of *M. bovis* BCG (<1%) was observed when the test was applied after PMS, confirming the high specificity (>99%) of the test for MAP if the optimized phage assay is applied in combination with PMS. Finally, no plaques

were observed for any of the milk bacteria tested, suggesting no risk of interference from bacterial species that might be encountered when testing raw milk for MAP.

Detection sensitivity, specifically LOD_{50}, was the last aspect of the PMS-phage assay investigated in the present study. Evidence to date suggests that MAP is typically present in raw milk in low numbers (Sweeney *et al.* 1992; Slana *et al.* 2009). Since conventional culture often does not have sufficient sensitivity (Slana *et al.* 2008a), an alternative detection method is required to rapidly demonstrate the presence of viable MAP in milk and achieve more accurate enumeration. Results from testing of both artificially and naturally infected milk samples indicate that the optimised PMS-phage assay possesses the sensitivity needed to detect low levels of MAP in raw milk. The optimised PMS-phage assay demonstrated higher sensitivity than both PMS-culture (on HEYM agar plates) and PMS-qPCR, targeting either IS900 or f57, when the test was used to test whole milk spiked at different levels with two MAP strains. Plaques were observed from all the milk samples, including those originally spiked with 1-10 MAP cells per 50 ml. Higher LOD_{50} were observed for the two other PMS-based detection methods (≥ 100 PFU per 50 ml). The higher MAP detection rates observed with the PMS-phage assay during raw milk testing further confirmed the higher sensitivity of this test compared to PMS-culture and PMS-qPCR. Viable MAP cells were detected by PMS-phage assay in 21.2% of individual milk samples and 59.1% of BTM samples tested. These detection rates were consistently higher than corresponding rates observed for PMS-IS900 qPCR (9.1% and 45.4%, respectively), PMS-f57qPCR (2.6% and 9.1%, respectively) and PMS-culture (11.6% and 50%, respectively). It should be noted that for raw milk testing MGIT broth was employed rather than HEYM agar plates for culture after PMS, and it was noticeable that the detection sensitivity of PMS-culture was much improved,

compared to the outcomes of LOD_{50} determinations using spiked milk. From past experience, liquid culture does tend to be more conducive for MAP growth than solid culture. The optimised PMS-phage assay proved to be a more sensitive and quicker alternative to conventional culture for demonstrating the presence of viable MAP in cows' milk.

Application of the optimised PMS-phage assay to test naturally infected milks during this study provided new information on numbers of viable MAP present in milk from individual animals and BTM from Johne's affected herds. Mean MAP counts estimated from individual infected cows and BTM milk were 228 PFU per 50 ml (range 6-948 PFU per 50 ml) and 144.9 PFU per 50 ml (range 18-685 PFU per 50 ml), respectively. These PMS-phage assay counts are higher than previous estimates of the numbers of MAP cells in both individual milk samples (2 to 8 CFU per 50 ml, Sweeney *et al.* 1992; <100 CFU per ml, Giese and Ahrens 2000; 4 to 20 CFU per 50 ml, Ayele *et al.* 2005; 10 to 560 cells per ml, Slana *et al.* 2008b) and BTM (1 to 9 cells per ml, Slana *et al.* 2008b and "several tens of cells per ml", Slana *et al.* 2009), which were obtained by either culture after chemical decontamination or real-time qPCR.

On the basis of the inclusivity, specificity, and sensitivity of the PMS-phage assay reported here, and its superior performance relative to PMS-qPCR and PMS-culture for testing raw milk samples, the PMS-phage assay represents the most sensitive test available to detect viable MAP in milk. However, as currently performed, the PMS-phage assay involves two overnight incubations, multiple timed steps and additions of reagents, and a plaque PCR to confirm MAP detection; so is laborious to carry out and it takes 2-3 days to obtain a MAP positive result. Only with considerable practice in application of the PMS-phage assay over several years

have consistent results been obtained within our laboratory. Whilst the phage amplification assay and PMS-phage assay are proving to be valuable MAP research tools, the PMS-phage assay is not suitable for adoption by the dairy industry as a milk screening test in its current format. The assay would require some streamlining, specifically to eliminate the need for the plaque assay and plaque PCR after phage amplification, in order to potentially become such a test. Potential avenues to achieve this goal are currently being explored in our laboratory.

To conclude, this study has demonstrated that the optimised PMS-phage assay is 100% inclusive for all MAP strains tested, >99% specific for MAP, and has a LOD_{50} of ~1 PFU per 50 ml milk. When used in combination with the optimized milk sample preparation protocol (Foddai and Grant, 2015), the assay detected viable MAP in raw milk from a substantial proportion (21.2%) of individual cows in a JD affected dairy herd (mean MAP count 228.1 PFU per 50 ml) and in 59.1% of BTM from JD affected dairy farms in Scotland (mean MAP count 136.83 PFU per 50 ml). This study has also demonstrated that the optimised PMS-phage assay is more sensitive than both PMS-qPCR and PMS-culture (on HEYM or in MGIT liquid medium). Unfortunately, in its present multi-step format, the PMS-phage assay does not represent a test that could be easily adopted by the dairy industry for routinely screening large numbers of milk samples to detect the presence of MAP. Efforts are continuing to streamline the assay, whilst maintaining its excellent detection sensitivity, to make it suitable for that purpose.

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Conflict of interest

No conflict of interest declared.

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Table 1. Details of *Mycobacterium* spp. tested to confirm specificity of the PMS-phage assay.

Test *Mycobacterium* sp.	Strain ID or Source
M. avium subsp. *avium*	AFBI[a]
M. avium subsp. *avium*	NCTC[b] 13034
M. bovis BCG	NCTC 5692
M. fortuitum	NCTC 10394
M. gordonae	NCTC 10267
M. intracellulare	NCTC 10425
M. kansasii	NCTC 10268
M. marinum	AFBI
M. scrofulaceum	AFBI
M. smegmatis	mc^2 155[c]
M. terrae	AFBI
M. xenopi	AFBI

[a] Culture kindly provided by Dr Lyanne McCallan, Veterinary Sciences Division, Agri-Food and Biosciences Institute (AFBI) for Northern Ireland, Belfast, UK. No other strain information available.

[b] Purchased from National Collection of Type Cultures, Colindale, London.

[c] Originally provided by Dr Ruth McNerney, London School of Hygiene and Tropical Medicine.

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Table 2. Comparison of limits of detection 50% (LOD_{50}) of the different PMS-based assays. Data represent number of samples test positive of total number of samples tested at each spiking level.

MAP strain	Test	Spiking level (PFU per 50 ml milk)				LOD_{50} (95% CI)
		10^2-10^3	10^1-10^2	1-10^1	Non-spiked	
NCTC 8578	PMS-phage assay	3/3	3/3	3/3	0/3	0.90 *
	PMS-IS900 qPCR	3/3	1/3	0/3	0/3	136.70 (21.40-872.90)
	PMS-f57 qPCR	3/3	0/3	0/3	0/3	303.70 *
	PMS-HEYM culture	0/3	0/3	0/3	0/3	- ǂ
ATCC 19698	PMS-phage assay	3/3	3/3	3/3	0/3	0.95 *
	PMS-IS900 qPCR	3/3	1/3	0/3	0/3	134.55 (23.45-772.80)
	PMS-f57 qPCR	3/3	0/3	0/3	0/3	291.00 *
	PMS-HEYM culture	0/3	0/3	0/3	0/3	-

* LOD_{50} could not be calculated because no spiking level yielded a partially positive response, i.e. <3/3

ǂ LOD_{50} could not be calculated because no milk sample at highest spiking level tested positive after PMS-culture.

Table 3. Number of MAP positive individual raw milk and bulk tank milk samples obtained by three PMS-based methods – optimised PMS-phage assay, PMS-qPCR (targeting both IS900 and F57) and PMS-culture in MGIT broth supplemented with PANTA and mycobactin J.

Type of milk sample	PMS-phage assay*			PMS-qPCR						PMS-MGIT culture †		
				IS900			F57					
	No. tested	No. Pos	% Pos	No. tested‡	No. Pos	% Pos	No. tested	No. Pos	% Pos	No. tested	No. Pos**	% Pos
Individual raw milk	146	31	21.2	77	7	9.1	77	2	2.6	146	17	11.6
Bulk tank milk	22	13	59.1	22	10	45.4	22	2	9.1	22	11	50

* Only reported PMS-phage assay positive when DNA harvested from plaques tested Plaque-PCR positive.

† Only reported PMS-culture positive if presence of MAP confirmed by IS900 PCR applied to broth culture.

‡ All 146 individual milk samples were not tested by either PMS-qPCR assay due to cost constraints. The 77 samples tested included 47 samples that had yielded plaques with the PMS-phage assay plus 30 other randomly selected PMS-phage assay negative samples.

Figure 1. Inclusivity of the optimised PMS-phage assay demonstrated by testing 43 different MAP strains, and variation in plaque counts obtained for duplicate tests on each strain. Data represent mean plaque count +/- standard deviation.

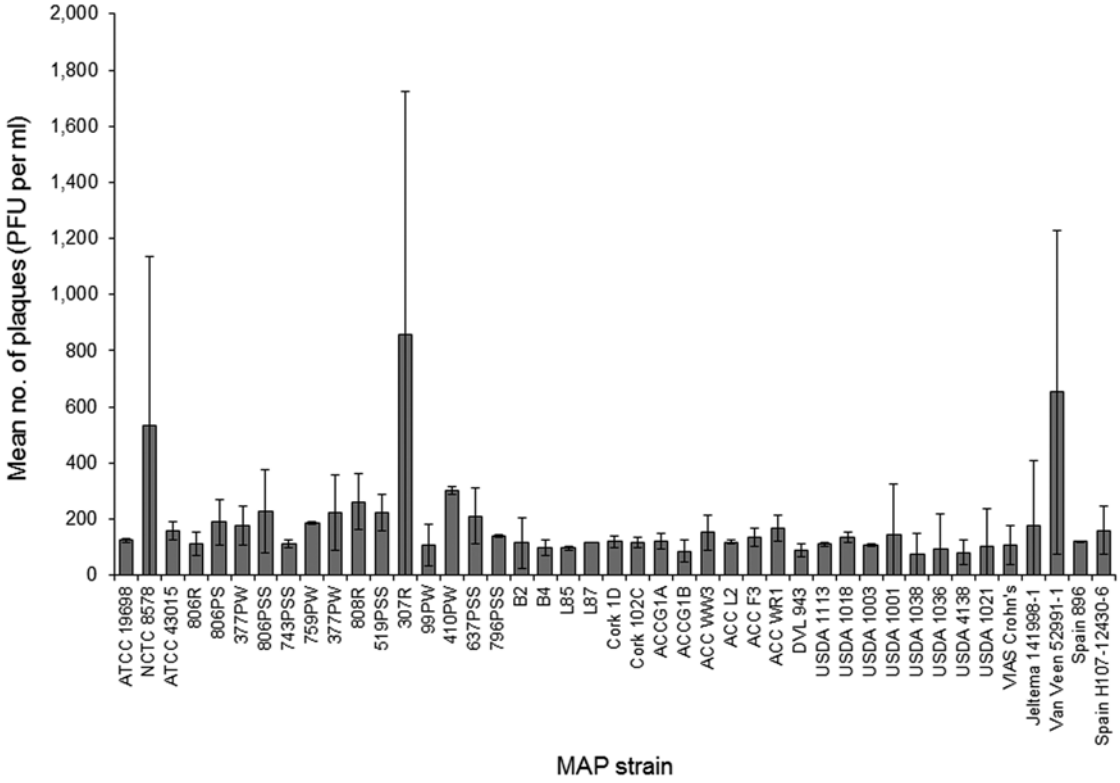

Figure 2. Venn diagrams showing inter-relationships between optimised PMS-phage assay, PMS-culture and PMS-IS900 qPCR results for individual raw milk samples (A and B) and BTM samples (C). An asterisk indicates that the corresponding PMS-phage assay yielded plaques but presence of MAP DNA was not confirmed by plaque-PCR, and so PMS-phage assay result was recorded as negative.