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# Diagnostic potential of the PMS-phage assay and PMS-culture to detect *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk samples

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Running title: PMS-based methods for detecting *MAP* in milk

**Summary**

Controlling the spread of Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), in domestic livestock is challenging. Current diagnostic methods lack sufficient sensitivity to detect sub-clinically infected animals, and thus better diagnostic methods are needed. This study was carried out to investigate the diagnostic potential of two novel peptide-mediated magnetic separation (PMS)-based tests - a PMS-phage assay and PMS-culture – both of which have been developed and optimised to detect viable *MAP* cells in bovine milk. Individual milk samples (50 ml) were obtained from 105 'non-infected' and 40 '*MAP*-infected' animals (classified as such on the basis of prior faecal culture and serum-ELISA results) in three dairy herds, and tested in parallel by the PMS-phage assay and PMS-culture. Diagnostic sensitivity (DSe) and specificity (DSp) of the PMS-phage and PMS-culture methods were determined relative to the MAP infection status of the animal contributing the milk sample. The PMS-based tests applied individually showed moderate DSe (PMS-culture 0.250 and PMS-phage assay 0.325) and high DSp (0.962 and 1.000, respectively). When results of the two PMS-based tests were combined, DSe increased substantially to 0.525 and the DSp was calculated to be 0.962. It was concluded that combined application of the PMS-phage assay and PMS-culture provided the most complete picture regarding the presence of viable *MAP* in bovine milk samples. A comprehensive validation of the PMS-based assays relative to currently used diagnostic methods (faecal culture and serum-ELISA) would be the next step in assessment of the diagnostic potential of these novel PMS-based methods.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*); peptide-mediated magnetic separation (PMS); milk; PMS-phage assay; PMS-culture; diagnosis; Johne's disease

## Introduction

Liquid and solid culture is still widely considered the 'gold standard' method for the detection of viable *MAP* in milk and faeces for the diagnosis of Johne's disease (Slana et al., 2008, Britton et al., 2016). However, due to the lack of a selective culture medium and the long incubation times required for the growth of *MAP*, contamination of cultures is common. Decontamination protocols are routinely employed prior to culture, however these can affect *MAP* viability resulting in non-isolation of some, or all, of the *MAP* present (Dundee et al. 2001, Bradner et al. 2013). Due to the problems associated with culturing *MAP* and the long waiting time for test results, *MAP* culture is an imperfect 'gold standard' for the detection of viable *MAP*. Other commonly used *MAP* diagnostic tools include ELISA-based methods, which have been extensively applied for the assessment of Johne's prevalence within herds (Slana et al., 2008). While ELISA methods are rapid, inexpensive and often highly specific, they are based on the detection of anti-*MAP* antibodies rather than viable *MAP* cells, so such tests identify whether an animal has sero-converted but do not necessarily confirm *MAP* infection. Thus, ELISA tests often possess low detection sensitivity (Lavers et al., 2014). The specificity of the serum-ELISA test may also be sub-optimal due to cross-reactivity with antibody responses to other environmental *Mycobacterium* spp. (Nielsen and Toft, 2008) or *Mycobacterium bovis* (Lilenbaum et al. 2007), depending on geographic circumstances

There is an urgent need for a rapid detection method for *MAP* which is both specific and sensitive, and which can distinguish between viable and non-viable cells. The recently developed Peptide-mediated magnetic separation (PMS)-phage assay, and/or PMS-culture, may represent such tests. Potentially, PMS could replace chemical decontamination prior to culture, thus avoiding deleterious effects on *MAP* viability that impair the analytical sensitivity of existing culture methods. The PMS-phage assay could speed-up the identification of *MAP* infected animals which are transmitting the infection through *MAP*-contaminated milk, which is key to limiting the spread of Johne's disease. Therefore, improvements in the sensitivity of *MAP* diagnostics would have a huge impact on the control of Johne's disease.

The PMS-based methods employ paramagnetic beads coated with *MAP*-specific biotinylated aMp3 and aMptD peptides (Foddai et al. 2010) to selectively capture and concentrate low numbers of *MAP* cells from milk samples. The analytical specificity of the PMS assay applied to milk was previously determined to be >98%, with an analytical sensitivity for the PMS-phage and PMS-culture assays of 10-$10^2$ pfu/ml and $10^2$-$10^3$ cfu/ml, respectively (O'Brien et al., 2016). These methods have principally been developed and optimised for detection of *MAP* in milk. Whilst the PMS-phage assay has been applied to faeces previously (Foddai et al. 2011), higher numbers of false positive plaques are generally encountered when testing faeces because the D29 phage involved can also infect various environmental *Mycobacterium* spp. (Rybniker et al. 2006) that may occur in that sample matrix. Also, given the higher background microflora of bovine faeces compared to milk, PMS alone prior to culture (without application of some form of mild chemical decontamination) is unlikely to be as successful when testing bovine faeces.

The present study was carried out to assess the diagnostic potential of the two PMS-based assays by testing milk samples from individual animals of known MAP infection status. Their diagnostic potential was assessed by determining diagnostic sensitivity (DSe), the proportion of animals with evidence of infection that test positive by the novel assay(s), and diagnostic specificity (DSp), the number of animals without evidence of infection that test negative by the novel assay(s). This study was not a validation study because the 'gold standard' tests (faecal culture and serum-ELISA) could not be carried out contemporaneously with the PMS-based tests for financial and logistical reasons.

## Materials and Methods

### Acquisition of milk samples

Milk samples were obtained from selected animals in three Northern Ireland dairy herds (A, B and C), for which previous faecal culture and serum-ELISA results were available to permit them to be categorised as 'MAP-infected' or 'non-infected for milk collection purposes. Herds

A and B were *MAP* infected dairy herds with test (faecal culture and/or serum ELISA) positive animals present, and both had a history of clinical Johne's cases. Herd C was a closed herd that had never had a known or suspected case of Johne's disease. In addition, all animals in the herd have consistently tested negative for *MAP* infection by faecal culture and ELISA in previous years. Animals in Herds A and B were deemed to be '*MAP* infected' if they had tested positive by faecal culture (using TREK automated ESPII liquid culture system) and/or serum-ELISA (IDEXX ELISA) when screened as part of the Department of Agriculture, Food and the Marine Food Institutional Research Measure-funded ICON*MAP* project in 2013. 'Non-infected' animals were those animals in Herd C that tested negative by both tests in 2013.

A total of 145 individual 50 ml milk samples (105 from 'non-infected' animals from Herd C and 40 from '*MAP* infected' animals in Herds A and B) were obtained from individual cows for testing by the PMS-phage assay and PMS-culture. It was hoped that the majority of milk samples from '*MAP* infected' cows would be sourced from animals positive by both faecal culture and serum-ELISA tests. However, due to the time lapse between blood and faecal testing in 2013 (no more recent *MAP* testing had been carried out) and milk sample collection between December 2015 and January 2016, many of the animals in this category had been culled from *MAP* infected herds A and B and were no longer available for milk sampling. In consequence, milk samples were mainly collected from infected animals positive by one or other of the diagnostic tests (nine positive by serum-ELISA only and 24 positive by faecal culture only) rather than both tests (n=7). The 105 milk samples from 'non-infected' cows were exclusively sourced from cows in the Johne's disease negative Herd C.

All milk samples were collected by a veterinarian during the early morning milking session on each farm. The vet was instructed to dip the teat of each animal in chlorhexidine and use an alcohol wipe to remove all visible dirt and faeces before milking each animal by hand into a pre-labelled sterile 50 ml centrifuge tube. Once collected, the milk samples were placed in a cool box with ice packs and kept at 4°C during transportation to the laboratory at Queen's University Belfast (QUB). Unless testing was to begin immediately, the milk samples

were stored at -80°C until required; as a previous study at QUB had found that *MAP* viability was not compromised if milk was stored for up to 4 weeks at -80°C (Foddai and Grant, 2015).

**Preparation of culture media**

Broth for PMS-culture was prepared using Middlebrook 7H9 broth (Difco), prepared according to the manufacturer's instructions, supplemented with 10 % (v/v) OADC (Difco) and 2 µg/ml mycobactin J (Synbiotics Europe SAS, Lyon, France) and PANTA (Polymyxin B 40,000 IU/l, Amphotericin B 4,000µg/l, Nalidixic acid 16,000 µg/l, Trimethoprim 4,000µg/l, and Azlocillin 4,000µg/l; Becton Dickinson, Oxford, UK). The autoclaved and supplemented 7H9 broth was dispensed in 5 ml volumes into 15 ml pre-sterilised screw-cap glass tubes that had not previously been used for *MAP* culture.

Herrold's egg yolk medium (HEYM) was prepared in-house using 9 g bacteriological peptone (Difco), 4.5 g NaCl, 15 g bacteriological agar, 2.7 g beef extract, 4.7 g sodium pyruvate and 27 ml glycerol (all from Sigma-Aldrich, Poole, UK), prepared in 900 ml water. After autoclaving at 121°C for 15 min and cooling to ~55°C the medium was supplemented with 2 µg/ml of Mycobactin J (Synbiotics Europe SAS), 5 ml of 2% (w/v) malachite green (Sigma-Aldrich) and 100 ml (v/v) sterile egg yolk (prepared in-house), before dispensing 5 ml volumes into 15 ml pre-sterilised screw-cap glass tubes that had not previously been used for *MAP* culture. The tubes were supported at an angle to allow the medium to solidify forming HEYM slopes. Both culture media were stored at 4°C until required.

**Peptide-mediated magnetic separation (PMS)-based tests**

The optimised milk testing procedure, described in detail previously by Foddai and Grant (2015), was adopted during this study. Frozen milk samples were thawed overnight at 4°C and brought to room temperature for at least an hour before centrifugation at 3,000 g for 15 min in a Rotina 380 centrifuge (Hettich, Germany). The milk fat and supernatant were discarded and the pellet re-suspended in 1 ml PBS pH 7.4 buffer containing 0.05 % (v/v)

166 Tween 20 (PBST). The samples were then ultrasonicated (pulse mode 37 kHz for 4 min in
167 ice-water) in the Ultrasonic PH 30 bath (Fisher Scientific Ltd) to break up any *MAP* clumps,
168 before automated magnetic separation was performed with 10 µl (5 µl of each coated bead)
169 of MyOne™ Tosylactivated® Dynabeads coated with biotinylated aMp3 and aMptD synthetic
170 peptides (coated in-house as described elsewhere, Foddai et al. 2010) using a Dynal
171 BeadRetriever (Invitrogen) and the pre-loaded 'Environmental' programme. This programme
172 consisted of a capture step for 30 min with constant mixing, two wash steps in 1 ml PBST for
173 1 min with mixing, and final resuspension of the beads in 1 ml 7H9 broth supplemented with
174 10% (v/v) OADC, 2 mM $CaCl_2$ and NOA antibiotic supplement (Nystatin 50,000 IU/l, Oxacillin
175 2 mg/l and Aztreonam 30 mg/l; Abtek Biologicals Ltd, Liverpool, UK). Sterile PBST was used
176 as a negative PMS control and a pure *MAP* cell suspension diluted in PBST as a positive
177 PMS control. Both controls were processed through the PMS-phage and PMS-culture assays
178 with each batch of test samples.

179 PMS-culture and confirmation of suspect positive cultures was carried out as follows:
180 of the 1 ml bead suspensions after PMS, 100 µl was inoculated into supplemented 7H9 broth
181 (described above) and 100 µl was spread onto HEYM agar slopes (described above). The
182 remaining 800 µl of bead suspension was processed through the phage amplification assay
183 (described below). The broth and HEYM slope PMS-cultures were incubated at 37°C and
184 absorbance measurements of the broths at $OD_{600nm}$ (measured using Biowave CO8000
185 Density meter, Biochrom Ltd., Cambridge, UK) were recorded periodically, as a measure of
186 microbial growth over time. Slopes were examined periodically for evidence of typical *MAP*
187 colonies.

188 Standard Ziehl-Neelsen (ZN) staining was used to determine if acid-fast bacteria were
189 present in PMS-culture broths showing an increase in $OD_{600\ nm}$ and in suspect colonies from
190 HEYM slopes. For broth cultures, 1 ml of broth was centrifuged for 10 min at 16,000 g
191 (Eppendorf 5424R). The supernatant was discarded and the pellet washed in 100 µl of
192 molecular grade water. The pellet was resuspended in 50 µl of molecular grade water. For
193 HEYM slopes the suspect *MAP* colonies were removed using a sterile loop and suspended

194 in 50 µl molecular grade water. Twenty microlitres of either sample was transferred onto
195 microscope slides and heat-fixed before ZN staining. The slides were examined under oil
196 immersion at 100x magnification.

197 The remainder of the 50 µl cell suspension from suspect *MAP* positive broths or
198 colonies was transferred to thin-walled PCR tubes and heated to 95°C for 25 min to lyse cells
199 and release DNA. The samples were centrifuged briefly to pellet cell debris before 5 µl of the
200 supernatant was added to 45 µl of PCR master mix containing 1X Green buffer, 3 mM $MgCl_2$,
201 200 µM of each dNTP, 1 U Platinum Taq (all from Invitrogen) and 20 pmol of P90
202 5'GTTCGGGGCCGTCGCTTAGG'3 and P91 5'GAGGTCGATCGCCCAC GTGA'3 (Moss et
203 al. 1992). The PCR cycling conditions were: 94°C for 2 min, 33 cycles of 94°C for 1 min,
204 62°C for 1 min, 72°C for 3 min, final extension was 72°C for 10 min before holding at 4°C.
205 The PCR products were visualised by agarose gel electrophoresis and the expected PCR
206 product size was 394 bp.

207 The PMS-phage assay was carried out essentially as described by Foddai et al.
208 (2011). After PMS of the milk samples, 800 µl of each bead suspension (in 7H9 broth
209 supplemented with 10 % (v/v) OADC, 2 mM $CaCl_2$ and NOA antibiotic supplement) was
210 processed through the phage amplification assay. Briefly, 100 µl of D29 phage solution
211 (containing approximately $10^8$ pfu) was added to each 800 µl sample and incubated for 2
212 hours at 37°C after which time 100 µl of freshly prepared 100 mM ferrous ammonium
213 sulphate (FAS) solution was added to each sample. The samples were mixed to ensure that
214 all of the internal surfaces of the vial were coated before being left to stand at room
215 temperature for 5 min. The samples were then vortexed and allowed to stand for a further 5
216 min before neutralising the sample by adding 5 ml of 7H9 broth containing 10% (v/v) OADC
217 and 2 mM $CaCl_2$. The samples were incubated at 37$^o$C until a total of 3.5 h had elapsed
218 since addition of D29 phages, after which the samples were transferred to Petri dishes
219 containing 1 ml (~$10^8$ cfu/ml) *Mycobacterium smegmatis* $mc^2$ 155 sensor cells and 5 ml
220 molten 7H9 agar. After gentle swirling to mix the plate contents, the plates were allowed to
221 solidify before incubation at 37°C overnight. The following morning, any plaques present

were counted and numbers recorded. A negative phage assay control (7H9 broth only) was included with each set of samples processed to ensure the efficacy of the FAS treatment to inactivate the seed D29 phage.

To confirm that the plaques observed were due to the presence of viable *MAP* in the original milk sample, and not arising from other *Mycobacterium* spp. or non-inactivated D29 phages, an IS*900*-based Plaque-PCR (Swift et al. 2014) was performed on DNA extracted from the plaques. Up to a maximum of 10 plaques per sample were selected for DNA extraction from each PMS-phage assay positive sample. The centre of each plaque was excised using a sterile loop and transferred to an Eppendorf tube. The DNA was extracted from the plaque using the Zymoclean™ Gel DNA Recovery kit (Cambridge Bioscience, UK), according to the manufacturer's instructions. DNA was eluted from the Zymoclean columns using 20 µl elution buffer (supplied with kit) or molecular grade water. DNA was stored at -20°C until required for plaque PCR. A protocol modified from Whittington et al. (1998) was used to target the IS*900* insertion element. To 40 µl of master mix containing 1 X DreamTaq Green Buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 U DreamTaq DNA polymerase (Thermo Fisher Scientific) and 250 ng of P90 5'GAAGGGTGTTCGGGGCCGTCGCTTAGG'3 and P91 5'GGCGTTGAGGTCGATCGCCC ACGTGAC'3 primers (Whittington et al. 1998), 10 µl of plaque DNA was added. The PCR cycling conditions were: 94°C for 5 min, 37 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min, final extension at 72°C for 4 min, and then sample cooled to 4°C. Agarose gel electrophoresis was used to visualise the PCR products and TrackIt™ 100 bp DNA ladder (Invitrogen) was loaded alongside the PCR products. The expected IS*900* PCR product size was 400 bp.

**Statistical analysis of results**

Statistical analysis was performed to compare PMS-phage and PMS-culture results with infection status of the animal supplying the milk samples (assigned on the basis of most recent faecal culture and serum-ELISA results). Kappa agreement was calculated between PMS-phage assay and PMS-culture results, and between results of these tests and the

250 animal's infection status, and Fisher's Exact Test was performed to obtain estimates of the
251 DSp and DSe of the PMS-phage assay, PMS-culture, and the combined PMS-based
252 methods, using GraphPad InStat (GraphPad Software Inc., La Jolla, CA, USA).
253
254 **Results**
255
256 **Performance of PMS-phage and PMS-culture assays**
257 A total of 145 milk samples from individual cattle were processed through the PMS-phage
258 assay and PMS-culture. Positive and negative control samples reported correctly throughout
259 the study; *MAP* B4 and ATCC 19698 broths were positive by the PMS-phage assay,
260 producing between 30-300 plaques which confirmed positive by *IS*900 Plaque-PCR, and
261 sterile broth was negative by the PMS-phage assay with no plaques observed. Only those
262 milk samples yielding plaques that were confirmed to be *MAP* positive by Plaque PCR were
263 deemed to be positive by the PMS-phage assay when results were statistically analysed. All
264 Herd C (*MAP* negative herd) milk samples tested negative by the PMS-phage assay. The
265 mean plaque counts for PMS-phage assay positive milk samples from Herd A and Herd B
266 (two MAP infected herds) were 94.9 pfu/50 ml and 8.0 pfu/50 ml, respectively (Table 1). Of
267 the 145 milk samples tested, 21 (14.5%) produced plaques by the PMS-phage assay, of
268 which 12 (8.3%) were confirmed to be *MAP* positive by Plaque *IS900* PCR (Table 1). One
269 milk sample (Herd A, milk sample 10 in Table 1) yielded just three plaques that tested
270 negative by IS*900* Plaque-PCR, so the PMS-phage assay result was recorded as negative,
271 but this sample subsequently tested PMS-culture positive. This Plaque-PCR result is likely
272 to represent a false negative PMS-phage assay result, perhaps due to the presence of a low
273 quantity of DNA being retrieved from just three plaques.
274 All *MAP* positive controls from the PMS-culture assay produced acid-fast positive broth
275 cultures, and suspect colonies that were confirmed to be *MAP* by IS*900* PCR. The negative
276 (sterile broth) controls did not demonstrate any evidence of acid-fast cells in broth cultures or
277 suspect colonies on HEYM slopes. PMS-culture was positive for 14 (9.7%) samples, i.e.

suspect colonies or broths confirmed by IS*900* PCR (Figure 1). Of the total number of PMS-phage assay and PMS-culture positives (n=25), only 2 (8.0%) samples were positive by both PMS tests (Figure 1). When the results of the PMS-phage and PMS-culture assays were analysed, Kappa agreement (± standard error) between the two tests was deemed to be 'poor' (0.061 ±0.099). The two tests, although detecting similar numbers of *MAP* positive milk samples, were seemingly detecting different subsets of infected animals.

**Diagnostic potential of PMS-phage and PMS-culture assays**

The ability of the PMS-phage, PMS-culture, and combined PMS-phage-culture assays to correctly identify '*MAP* infected' and 'non-infected' animals from Northern Ireland dairy herds was assessed. The infection status of each animal from which milk was obtained had been assigned on the basis of a positive faecal culture and/or a positive serum-ELISA result at the time of last *MAP* testing, which was in 2013. Contingency tables (2x2) of results were constructed separately for PMS-phage assay and PMS-culture, and also for the combined PMS-based tests (Table 2). Of the 40 *MAP* 'infected' animals, the PMS-phage assay identified 13 (32.5%) animals shedding viable *MAP* in milk which was deemed to be 'moderate' agreement, with a corresponding Kappa value (± standard error) of 0.411 ± 0.083 (Table 2). The DSp of the PMS-phage assay was calculated to be 1.000 and DSe to be 0.325 (Table 2). In contrast, the PMS-culture assay was found to have 'fair' agreement with animal infection status (Kappa value ± standard error of 0.265 ± 0.084). PMS-culture detected viable *MAP* in the milk of 10 (25%) of 40 '*MAP* infected' animals, thereby demonstrating a DSe of 0.250 (Table 3). Diagnostic specificity (DSp) of PMS-culture was 0.962 because 4 (3.8%) of 105 animals categorised as 'non-infected' animals tested positive for viable *MAP* by this test. When results of the PMS-phage and PMS-culture assays were combined, the agreement with animal infection status was 'moderate' with a Kappa value of 0.551 ± 0.080, which was greater agreement than either test individually. Just over half of the '*MAP* infected' animals were correctly identified when the assays were combined, increasing DSe to 0.525; which is much higher than for either the PMS-phage assay (0.325)

306 or PMS-culture (0.250) applied separately (Table 2). Diagnostic specificity of the combined
307 PMS-based tests remained high at 0.962.
308
309 **Discussion**
310 The accurate identification of cattle shedding viable *MAP* in their milk is crucial to preventing
311 transmission of Johne's disease between cows and calves. Detection of viable *MAP* in cows'
312 milk more generally is also of interest from a public health-food safety perspective. The
313 objective of this study was to evaluate the diagnostic potential of two novel PMS-based
314 assays, specifically as tests to detect infected animals shedding viable *MAP* in their milk.
315 Previously published studies have detected 10-$10^3$ *MAP*/50 ml milk by decontamination and
316 culture (Sweeney et al., 1992, Giese and Ahrens, 2000, Ayele et al., 2005) and $10^2$-$10^4$
317 *MAP*/50 ml milk by qPCR (Slana et al., 2008) or IS*900* PCR (Stabel et al., 2014). The PMS-
318 phage assay and PMS-culture have sufficient analytical sensitivity (10-$10^2$ pfu/ml and $10^2$-$10^3$
319 cfu/ml, respectively; O'Brien et al., 2016) to be able to detect these low numbers of *MAP* in
320 cow's milk. However, it is documented that *MAP* shedding into milk can be intermittent
321 (Fecteau and Whitlock, 2010), and the number of *MAP* shed can vary by stage of infection,
322 with clinical animals shedding higher numbers of *MAP* in their milk than sub-clinical animals
323 (Sweeney et al., 1992, Stabel et al., 2014). Stage of lactation and season are also influential
324 factors in the accurate diagnosis of Johne's disease. For example, *MAP* shedding primarily
325 occurs during early lactation (Nielson and Toft, 2012, Zervens et al., 2013, Stabel et al.,
326 2014), and spring and summer are thought to be the seasons which offer the greatest
327 chance of identifying *MAP* infected animals (Wolf et al., 2015). Milk samples tested during
328 this study were collected between November 2015 and January 2016, which may have
329 negatively impacted the number of *MAP* positive samples detected. It should also be noted
330 that the number of *MAP* shed directly into milk within the udder of infected animals can also
331 be significantly augmented by faecal contamination during the milking process (Vissers et al.
332 2007). Whilst teat cleaning was carried out by the vet prior to collection of a milk sample from
333 each animal during this study, the efficacy or consistency of this decontamination step

cannot be guaranteed. Consequently, some of the *MAP* contamination of milk detected during this study may have originated from faeces of infected animals rather than due to direct shedding of *MAP* into milk within the udder. Irrespective of the potential source of *MAP* in milk, a method to accurately detect the presence of viable *MAP* in milk is urgently required.

During this study, a total of 145 milk samples from '*MAP* infected' (n=40) and 'non-infected' dairy cows (n=105) were tested by both the PMS-phage assay and PMS-culture. Overall, there was 'poor' agreement between results of the two PMS-based tests (Kappa ± SE of 0.061 ± 0.099). Approximately equal numbers of animals, but different subsets of animals, tested positive for viable *MAP* by the two PMS-based tests (Figure 1), which was an unexpected finding. There are a couple of possible explanations. Firstly, *MAP* cells present in raw milk may exist in different metabolic states (viable, viable but non culturable, dormant, dead), and this will have influenced *MAP* cell infectivity by the D29 phage (Swift et al., 2014). The PMS-phage assay will only detect *MAP* cells that are fully viable at the point of testing, since the D29 phage needs to 'hijack' the *MAP* cell in order to replicate itself. If the phage is unable to replicate, then the *MAP* cell will not burst and release D29 phages to initiate plaque formation in the plaque assay and false negative PMS-phage assay results will be obtained. Conversely, if the *MAP* cells present are viable but non culturable at the point of testing, a positive PMS-phage assay result may be obtained that may or may not subsequently be backed up by a PMS-culture positive result. During culture *MAP* cells will have the opportunity to recover full viability during the long incubation period, resulting in PMS-culture positives even though the corresponding PMS-phage assay result may have been negative. A second, more practical explanation for lack of agreement between the PMS-phage assay and PMS-culture results could be the fact that the bead sample after PMS was unequally split between the two detection methods. The majority (800 µl) of each 1 ml bead suspension after PMS was processed through the phage assay and only 200 µl was cultured. This may explain some of the discrepancies between the PMS-phage and PMS-culture results, particularly if milk samples containing low numbers of *MAP* were being testing. Milk samples containing higher numbers of *MAP* would not have been so greatly impacted by the unequal

362 splitting of the milk sample, as these would likely still be positive for *MAP* in both fractions
363 tested. Due to the finding of 'poor' agreement between results of the PMS-phage assay and
364 PMS-culture, it was decided that it may be more appropriate to combine the test results when
365 assessing diagnostic potential of the PMS-based assays. Diagnostic sensitivity (DSe)
366 increased from 0.250 (PMS-culture) and 0.325 (PMS-phage assay) to 0.525 when results of
367 the two PMS-based tests were combined. DSp of the combined PMS-based methods was
368 the same as PMS-culture (0.962), which was lower than for the PMS-phage assay (1.000).
369 The DSp and DSe of the PMS-phage assay, PMS-culture, and the combined PMS-
370 based assays, were determined relative to the *MAP* infection status of the animals supplying
371 the milk samples (Table 2). There was a two year gap between faecal culture and serum-
372 ELISA testing of the animals in 2013, upon which each animal's infection status was
373 assigned, and the collection of milk samples for PMS-phage assay and PMS-culture testing
374 in December 2015 and January 2016. This situation was not ideal, but was unavoidable
375 given that regular Johne's testing of cattle in these Northern Ireland dairy herds was not
376 carried out. The infection status of the '*MAP* infected' animals in Herds A and B from which
377 milk was collected is unlikely to have changed in the intervening years (other than due to
378 Johne's disease progression), so estimates of DSe obtained for the PMS-based tests should
379 still be reliable. For example, PMS-culture of milk demonstrated a DSe of 0.250, which is
380 similar to that estimated for faecal culture (0.20) of Irish cattle by More et al. (2013); DSe of
381 the PMS-phage assay and combined PMS-based tests were higher (0.325 and 0.525,
382 respectively). The infection status of the animals in negative Herd C could have changed
383 between 2013 and 2015/16. However, according to the animal health database of the
384 Department of Agriculture, Environment and Rural Affairs for Northern Ireland, Herd C is still
385 officially recorded as MAP 'non-infected' (Dr Lyanne McCallan, Agri-Food and Biosciences
386 Institute for Northern Ireland, personal communication). PMS-culture results suggest there
387 were four cows in negative Herd C that may have been *MAP*-infected at the time of milk
388 collection in 2015/16. Since these milk samples were PMS-culture positive, and not just
389 PMS-phage assay positive, it seems rather unlikely they could be false positive results. The

390 detection of viable *MAP* in some milk samples from supposedly 'non-infected' animals
391 reduced the DSp estimate from 1.000 for the PMS-phage assay to 0.962 for PMS-culture
392 and for the combined use of the PMS-based tests (Table 2).

393 On the basis of the results of this study, the combined application of the PMS-phage
394 assay and PMS-culture would appear to provide the most complete picture about *MAP*
395 contamination of the milk of infected cows; DSp of the combined tests is high (0.962) and
396 DSe is moderately high (0.525). A properly designed validation study, testing milk samples
397 contemporaneously with faeces and blood samples from the same animals, would be the
398 next step in order to accurately assess the diagnostic potential of the novel PMS-based
399 assays and their performance relative to the currently used diagnostic tests (faecal culture
400 and serum-ELISA).

401

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501 **Table 1.** PMS-phage assay results for milk samples collected from *MAP* infected animals in
502 Herds A and B. Only milk samples yielding plaques (pfu) are listed; all other milk samples
503 tested negative by the PMS-phage assay.

| Herd ID | Milk Sample ID | Plaque count (pfu/50 ml) | IS900 Plaque-PCR result* |
|---|---|---|---|
| A | 2‡ | 13 | positive |
| A | 6 | 2 | positive |
| A | 10‡ | 3 | negative |
| A | 17 | 24 | positive |
| A | 32‡ | 2 | positive |
| A | 36 | >300 | positive |
| A | 37 | 4 | positive |
| A | 44 | >300 | negative |
| A | 45 | 2 | positive |
| A | 46 | >300 | positive |
| A | 47 | >300 | positive |
| A | 53 | 2 | positive |
| B | 1 | 6 | negative |
| B | 3 | 9 | negative |
| B | 4 | 2 | negative |
| B | 8 | 3 | negative |
| B | 12 | 25 | negative |
| B | 16 | 15 | positive |
| B | 17 | 1 | positive |
| B | 21 | 3 | negative |
| B | 24 | 4 | negative |

504 * Plaque PCR result dictated PMS-phage assay result - if the presence of MAP DNA was not
505 confirmed the result was PMS-phage assay negative.
506 ‡ Milk samples that subsequently tested PMS-culture positive, one of which had a negative
507 PMS-phage assay result (Herd A, sample 10).
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**Table 2.** 2 x 2 contingency tables comparing results of PMS-phage assay, PMS-culture and combined PMS methods with *MAP* infection status of cattle providing the milk samples tested. Kappa agreement and estimates of diagnostic sensitivity and specificity, determined using Fisher's Exact Test, are presented for each of the tests.

| Test | Test result | Infection status* | | *Kappa* statistic ± SE | Diagnostic sensitivity (95% CI) | Diagnostic specificity (95% CI) |
|---|---|---|---|---|---|---|
| | | *MAP* infected‡ (n=40) | Non infected‡ (n=105) | | | |
| PMS-phage assay | Positive | 13 | 0 | 0.411 ± 0.083 ('moderate') | 0.325 (0.186, 0.491) | 1.000 (0.966, 1.000) |
| | Negative | 27 | 105 | | | |
| PMS-culture | Positive | 10 | 4 | 0.265 ± 0.084 ('fair') | 0.250 (0.127, 0.412) | 0.962 (0.905, 0.989) |
| | Negative | 30 | 101 | | | |
| Combined PMS methods | Positive | 21 | 4 | 0.551 ± 0.080 ('moderate') | 0.525 (0.361, 0.685) | 0.962 (0.905, 0.989) |
| | Negative | 19 | 101 | | | |

* Infection status of cow was assigned, prior to milk collection, based on results of faecal culture and serum-ELISA testing performed in 2013.

‡ The 40 '*MAP* infected' cows were in Johne's affected Herds A and B. The 105 'non-infected' cows were exclusively in *MAP* negative Herd C.

**Figure Legends**

**Fig. 1.** Venn diagram showing inter-relationships between results of the PMS-phage assay and PMS-culture applied to 145 bovine milk samples.