

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

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5	L. M. O'Brien ¹ , C. G. McAloon ² , L. D. Stewart ¹ , S. A. J. Strain ³ and I. R. Grant ^{1*}					
6						
7	¹ Institute for Global Food Security, School of Biological Sciences, Queen's University					
8	Belfast, Northern Ireland, United Kingdom.					
9	² Section of Herd Health and Animal Husbandry, School of Veterinary Medicine, University					
10	College Dublin, Dublin, Ireland.					
11	^c Animal Health and Welfare Northern Ireland, Dungannon, Northern Ireland, United					
12	Kingdom.					
13						
14	*Correspondence: I.R. Grant, Institute for Global Food Security, School of Biological					
15	Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9					
16	7BL, Northern Ireland, United Kingdom. Tel: +44 28 9097 2109, i.grant@qub.ac.uk					
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18	Running title: PMS-based methods for detecting MAP in milk					
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26 Summary

Controlling the spread of Johne's disease, caused by Mycobacterium avium subsp. 27 paratuberculosis (MAP), in domestic livestock is challenging. Current diagnostic methods 28 lack sufficient sensitivity to detect sub-clinically infected animals, and thus better diagnostic 29 30 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 31 PMS-culture – both of which have been developed and optimised to detect viable MAP cells 32 in bovine milk. Individual milk samples (50 ml) were obtained from 105 'non-infected' and 40 33 'MAP-infected' animals (classified as such on the basis of prior faecal culture and serum-34 ELISA results) in three dairy herds, and tested in parallel by the PMS-phage assay and 35 PMS-culture. Diagnostic sensitivity (DSe) and specificity (DSp) of the PMS-phage and PMS-36 37 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 38 DSe (PMS-culture 0.250 and PMS-phage assay 0.325) and high DSp (0.962 and 1.000, 39 40 respectively). When results of the two PMS-based tests were combined, DSe increased 41 substantially to 0.525 and the DSp was calculated to be 0.962. It was concluded that 42 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 43 44 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 45 of these novel PMS-based methods. 46

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

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54 Introduction

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Liquid and solid culture is still widely considered the 'gold standard' method for the detection 56 of viable MAP in milk and faeces for the diagnosis of Johne's disease (Slana et al., 2008, 57 58 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. 59 Decontamination protocols are routinely employed prior to culture, however these can affect 60 MAP viability resulting in non-isolation of some, or all, of the MAP present (Dundee et al. 61 2001, Bradner et al. 2013). Due to the problems associated with culturing MAP and the long 62 waiting time for test results. MAP culture is an imperfect 'gold standard' for the detection of 63 viable MAP. Other commonly used MAP diagnostic tools include ELISA-based methods, 64 which have been extensively applied for the assessment of Johne's prevalence within herds 65 66 (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 67 68 tests identify whether an animal has sero-converted but do not necessarily confirm MAP 69 infection. Thus, ELISA tests often possess low detection sensitivity (Lavers et al., 2014). The 70 specificity of the serum-ELISA test may also be sub-optimal due to cross-reactivity with 71 antibody responses to other environmental Mycobacterium spp. (Nielsen and Toft, 2008) or Mycobacterium bovis (Lilenbaum et al. 2007), depending on geographic circumstances 72

There is an urgent need for a rapid detection method for MAP which is both specific 73 and sensitive, and which can distinguish between viable and non-viable cells. The recently 74 developed Peptide-mediated magnetic separation (PMS)-phage assay, and/or PMS-culture, 75 may represent such tests. Potentially, PMS could replace chemical decontamination prior to 76 culture, thus avoiding deleterious effects on MAP viability that impair the analytical sensitivity 77 of existing culture methods. The PMS-phage assay could speed-up the identification of MAP 78 infected animals which are transmitting the infection through MAP-contaminated milk, which 79 is key to limiting the spread of Johne's disease. Therefore, improvements in the sensitivity of 80 81 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 82 biotinylated aMp3 and aMptD peptides (Foddai et al. 2010) to selectively capture and 83 concentrate low numbers of MAP cells from milk samples. The analytical specificity of the 84 PMS assay applied to milk was previously determined to be >98%, with an analytical 85 86 sensitivity for the PMS-phage and PMS-culture assays of 10-10² pfu/ml and 10²-10³ cfu/ml, respectively (O'Brien et al., 2016). These methods have principally been developed and 87 optimised for detection of MAP in milk. Whilst the PMS-phage assay has been applied to 88 faeces previously (Foddai et al. 2011), higher numbers of false positive plagues are generally 89 encountered when testing faeces because the D29 phage involved can also infect various 90 environmental Mycobacterium spp. (Rybniker et al. 2006) that may occur in that sample 91 92 matrix. Also, given the higher background microflora of bovine faeces compared to milk, 93 PMS alone prior to culture (without application of some form of mild chemical 94 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-95 96 based assays by testing milk samples from individual animals of known MAP infection status. 97 Their diagnostic potential was assessed by determining diagnostic sensitivity (DSe), the 98 proportion of animals with evidence of infection that test positive by the novel assay(s), and 99 diagnostic specificity (DSp), the number of animals without evidence of infection that test 100 negative by the novel assay(s). This study was not a validation study because the 'gold 101 standard' tests (faecal culture and serum-ELISA) could not be carried out contemporaneously with the PMS-based tests for financial and logistical reasons. 102

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104 Materials and Methods

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106 Acquisition of milk samples

107 Milk samples were obtained from selected animals in three Northern Ireland dairy herds (A, B 108 and C), for which previous faecal culture and serum-ELISA results were available to permit 109 them to be categorised as 'MAP-infected' or 'non-infected for milk collection purposes. Herds

110 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 111 closed herd that had never had a known or suspected case of Johne's disease. In addition, 112 all animals in the herd have consistently tested negative for MAP infection by faecal culture 113 114 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 115 system) and/or serum-ELISA (IDEXX ELISA) when screened as part of the Department of 116 117 Agriculture, Food and the Marine Food Institutional Research Measure-funded ICONMAP 118 project in 2013. 'Non-infected' animals were those animals in Herd C that tested negative by both tests in 2013. 119

120 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 121 testing by the PMS-phage assay and PMS-culture. It was hoped that the majority of milk 122 samples from 'MAP infected' cows would be sourced from animals positive by both faecal 123 culture and serum-ELISA tests. However, due to the time lapse between blood and faecal 124 125 testing in 2013 (no more recent MAP testing had been carried out) and milk sample 126 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 127 sampling. In consequence, milk samples were mainly collected from infected animals 128 positive by one or other of the diagnostic tests (nine positive by serum-ELISA only and 24 129 positive by faecal culture only) rather than both tests (n=7). The 105 milk samples from 'non-130 infected' cows were exclusively sourced from cows in the Johne's disease negative Herd C. 131

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).

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142 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/I, Amphotericin B 4,000µg/I, Nalidixic acid 16,000 µg/I, Trimethoprim 4,000µg/I, and Azlocillin 4,000µg/I; 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 150 151 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. 152 153 After autoclaving at 121°C for 15 min and cooling to ~55°C the medium was supplemented 154 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 155 volumes into 15 ml pre-sterilised screw-cap glass tubes that had not previously been used 156 for MAP culture. The tubes were supported at an angle to allow the medium to solidify 157 forming HEYM slopes. Both culture media were stored at 4°C until required. 158

159

160 **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 ice-water) in the Ultrasonic PH 30 bath (Fisher Scientific Ltd) to break up any MAP clumps, 167 before automated magnetic separation was performed with 10 µl (5 µl of each coated bead) 168 of MyOne[™] Tosylactivated[®] Dynabeads coated with biotinylated aMp3 and aMptD synthetic 169 170 peptides (coated in-house as described elsewhere, Foddai et al. 2010) using a Dynal BeadRetriever (Invitrogen) and the pre-loaded 'Environmental' programme. This programme 171 consisted of a capture step for 30 min with constant mixing, two wash steps in 1 ml PBST for 172 1 min with mixing, and final resuspension of the beads in 1 ml 7H9 broth supplemented with 173 10% (v/v) OADC, 2 mM CaCl₂ and NOA antibiotic supplement (Nystatin 50,000 IU/I, Oxacillin 174 175 2 mg/l and Aztreonam 30 mg/l; Abtek Biologicals Ltd, Liverpool, UK). Sterile PBST was used as a negative PMS control and a pure MAP cell suspension diluted in PBST as a positive 176 PMS control. Both controls were processed through the PMS-phage and PMS-culture assays 177 178 with each batch of test samples.

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

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

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

The remainder of the 50 µl cell suspension from suspect MAP positive broths or 197 198 colonies was transferred to thin-walled PCR tubes and heated to 95°C for 25 min to lyse cells and release DNA. The samples were centrifuged briefly to pellet cell debris before 5 µl of the 199 supernatant was added to 45 µl of PCR master mix containing 1X Green buffer, 3 mM MgCl₂, 200 200 µM of each dNTP, 1 U Platinum Taq (all from Invitrogen) and 20 pmol of P90 201 5'GTTCGGGGCCGTCGCTTAGG'3 and P91 5'GAGGTCGATCGCCCAC GTGA'3 (Moss et 202 al. 1992). The PCR cycling conditions were: 94°C for 2 min, 33 cycles of 94°C for 1 min, 203 62°C for 1 min, 72°C for 3 min, final extension was 72°C for 10 min before holding at 4°C. 204 205 The PCR products were visualised by agarose gel electrophoresis and the expected PCR 206 product size was 394 bp.

The PMS-phage assay was carried out essentially as described by Foddai et al. 207 (2011). After PMS of the milk samples, 800 µl of each bead suspension (in 7H9 broth 208 209 supplemented with 10 % (v/v) OADC, 2 mM CaCl₂ and NOA antibiotic supplement) was processed through the phage amplification assay. Briefly, 100 µl of D29 phage solution 210 (containing approximately 10⁸ pfu) was added to each 800 µl sample and incubated for 2 211 hours at 37°C after which time 100 µl of freshly prepared 100 mM ferrous ammonium 212 sulphate (FAS) solution was added to each sample. The samples were mixed to ensure that 213 all of the internal surfaces of the vial were coated before being left to stand at room 214 temperature for 5 min. The samples were then vortexed and allowed to stand for a further 5 215 min before neutralising the sample by adding 5 ml of 7H9 broth containing 10% (v/v) OADC 216 and 2 mM CaCl₂. The samples were incubated at 37°C until a total of 3.5 h had elapsed 217 since addition of D29 phages, after which the samples were transferred to Petri dishes 218 containing 1 ml (~10⁸ cfu/ml) Mycobacterium smegmatis mc² 155 sensor cells and 5 ml 219 molten 7H9 agar. After gentle swirling to mix the plate contents, the plates were allowed to 220 221 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 225 226 original milk sample, and not arising from other Mycobacterium spp. or non-inactivated D29 phages, an IS900-based Plague-PCR (Swift et al. 2014) was performed on DNA extracted 227 from the plaques. Up to a maximum of 10 plaques per sample were selected for DNA 228 extraction from each PMS-phage assay positive sample. The centre of each plaque was 229 excised using a sterile loop and transferred to an Eppendorf tube. The DNA was extracted 230 from the plaque using the Zymoclean[™] Gel DNA Recovery kit (Cambridge Bioscience, UK), 231 according to the manufacturer's instructions. DNA was eluted from the Zymoclean columns 232 using 20 µl elution buffer (supplied with kit) or molecular grade water. DNA was stored at -233 20°C until required for plaque PCR. A protocol modified from Whittington et al. (1998) was 234 used to target the IS900 insertion element. To 40 µl of master mix containing 1 X DreamTaq 235 Green Buffer, 2.5 mM MgCl₂, 200 µM of each dNTP, 1 U DreamTag DNA polymerase 236 237 (Thermo Fisher Scientific) and 250 ng of P90 5'GAAGGGTGTTCGGGGCCGTCGCTTAGG'3 238 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 239 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 240 then sample cooled to 4°C. Agarose gel electrophoresis was used to visualise the PCR 241 products and TrackIt[™] 100 bp DNA ladder (Invitrogen) was loaded alongside the PCR 242 products. The expected IS900 PCR product size was 400 bp. 243

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245 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

animal's infection status, and Fisher's Exact Test was performed to obtain estimates of the
DSp and DSe of the PMS-phage assay, PMS-culture, and the combined PMS-based
methods, using GraphPad InStat (GraphPad Software Inc., La Jolla, CA, USA).

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254 Results

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256 Performance of PMS-phage and PMS-culture assays

A total of 145 milk samples from individual cattle were processed through the PMS-phage 257 assay and PMS-culture. Positive and negative control samples reported correctly throughout 258 the study; MAP B4 and ATCC 19698 broths were positive by the PMS-phage assay, 259 producing between 30-300 plaques which confirmed positive by IS900 Plaque-PCR, and 260 261 sterile broth was negative by the PMS-phage assay with no plaques observed. Only those milk samples yielding plaques that were confirmed to be MAP positive by Plaque PCR were 262 deemed to be positive by the PMS-phage assay when results were statistically analysed. All 263 264 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 (two MAP infected herds) were 94.9 pfu/50 ml and 8.0 pfu/50 ml, respectively (Table 1). Of 266 267 the 145 milk samples tested, 21 (14.5%) produced plaques by the PMS-phage assay, of which 12 (8.3%) were confirmed to be MAP positive by Plaque IS900 PCR (Table 1). One 268 269 milk sample (Herd A, milk sample 10 in Table 1) yielded just three plaques that tested 270 negative by IS900 Plaque-PCR, so the PMS-phage assay result was recorded as negative, but this sample subsequently tested PMS-culture positive. This Plaque-PCR result is likely 271 to represent a false negative PMS-phage assay result, perhaps due to the presence of a low 272 quantity of DNA being retrieved from just three plaques. 273

All *MAP* positive controls from the PMS-culture assay produced acid-fast positive broth cultures, and suspect colonies that were confirmed to be *MAP* by IS*900* PCR. The negative (sterile broth) controls did not demonstrate any evidence of acid-fast cells in broth cultures or 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 PMSphage 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 (\pm standard error) between the two tests was deemed to be 'poor' (0.061 \pm 0.099). The two tests, although detecting similar numbers of *MAP* positive milk samples, were seemingly detecting different subsets of infected animals.

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

The ability of the PMS-phage, PMS-culture, and combined PMS-phage-culture assays to 286 correctly identify 'MAP infected' and 'non-infected' animals from Northern Ireland dairy herds 287 288 was assessed. The infection status of each animal from which milk was obtained had been 289 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 290 constructed separately for PMS-phage assay and PMS-culture, and also for the combined 291 PMS-based tests (Table 2). Of the 40 MAP 'infected' animals, the PMS-phage assay 292 293 identified 13 (32.5%) animals shedding viable MAP in milk which was deemed to be 294 'moderate' agreement, with a corresponding Kappa value (\pm standard error) of 0.411 \pm 0.083 295 (Table 2). The DSp of the PMS-phage assay was calculated to be 1.000 and DSe to be 296 0.325 (Table 2). In contrast, the PMS-culture assay was found to have 'fair' agreement with 297 animal infection status (Kappa value ± standard error of 0.265 ± 0.084). PMS-culture 298 detected viable MAP in the milk of 10 (25%) of 40 'MAP infected' animals, thereby 299 demonstrating a DSe of 0.250 (Table 3). Diagnostic specificity (DSp) of PMS-culture was 300 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 301 combined, the agreement with animal infection status was 'moderate' with a Kappa value of 302 0.551 ± 0.080, which was greater agreement than either test individually. Just over half of 303 the 'MAP infected' animals were correctly identified when the assays were combined, 304 305 increasing DSe to 0.525; which is much higher than for either the PMS-phage assay (0.325)

or PMS-culture (0.250) applied separately (Table 2). Diagnostic specificity of the combined
 PMS-based tests remained high at 0.962.

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309 Discussion

310 The accurate identification of cattle shedding viable MAP in their milk is crucial to preventing transmission of Johne's disease between cows and calves. Detection of viable MAP in cows' 311 milk more generally is also of interest from a public health-food safety perspective. The 312 objective of this study was to evaluate the diagnostic potential of two novel PMS-based 313 assays, specifically as tests to detect infected animals shedding viable MAP in their milk. 314 Previously published studies have detected 10-10³ MAP/50 ml milk by decontamination and 315 culture (Sweeney et al., 1992, Giese and Ahrens, 2000, Ayele et al., 2005) and 10²-10⁴ 316 MAP/50 ml milk by qPCR (Slana et al., 2008) or IS900 PCR (Stabel et al., 2014). The PMS-317 phage assay and PMS-culture have sufficient analytical sensitivity (10-10² pfu/ml and 10²-10³ 318 cfu/ml, respectively; O'Brien et al., 2016) to be able to detect these low numbers of MAP in 319 320 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 (Sweeney et al., 1992, Stabel et al., 2014). Stage of lactation and season are also influential 323 factors in the accurate diagnosis of Johne's disease. For example, MAP shedding primarily 324 325 occurs during early lactation (Nielson and Toft, 2012, Zervens et al., 2013, Stabel et al., 2014), and spring and summer are thought to be the seasons which offer the greatest 326 chance of identifying MAP infected animals (Wolf et al., 2015). Milk samples tested during 327 this study were collected between November 2015 and January 2016, which may have 328 negatively impacted the number of MAP positive samples detected. It should also be noted 329 that the number of MAP shed directly into milk within the udder of infected animals can also 330 be significantly augmented by faecal contamination during the milking process (Vissers et al. 331 2007). Whilst teat cleaning was carried out by the vet prior to collection of a milk sample from 332 333 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 334 during this study may have originated from faeces of infected animals rather than due to 335 direct shedding of MAP into milk within the udder. Irrespective of the potential source of MAP 336 in milk, a method to accurately detect the presence of viable MAP in milk is urgently required. 337 338 During this study, a total of 145 milk samples from 'MAP infected' (n=40) and 'noninfected' dairy cows (n=105) were tested by both the PMS-phage assay and PMS-culture. 339 Overall, there was 'poor' agreement between results of the two PMS-based tests (Kappa ± 340 341 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 342 unexpected finding. There are a couple of possible explanations. Firstly, MAP cells present 343 344 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). 345 The PMS-phage assay will only detect MAP cells that are fully viable at the point of testing, 346 since the D29 phage needs to 'hijack' the MAP cell in order to replicate itself. If the phage is 347 348 unable to replicate, then the MAP cell will not burst and release D29 phages to initiate plaque 349 formation in the plaque assay and false negative PMS-phage assay results will be obtained. 350 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 351 backed up by a PMS-culture positive result. During culture MAP cells will have the 352 opportunity to recover full viability during the long incubation period, resulting in PMS-culture 353 positives even though the corresponding PMS-phage assay result may have been negative. 354 A second, more practical explanation for lack of agreement between the PMS-phage assay 355 and PMS-culture results could be the fact that the bead sample after PMS was unequally 356 split between the two detection methods. The majority (800 µl) of each 1 ml bead suspension 357 after PMS was processed through the phage assay and only 200 µl was cultured. This may 358 explain some of the discrepancies between the PMS-phage and PMS-culture results, 359 particularly if milk samples containing low numbers of MAP were being testing. Milk samples 360 361 containing higher numbers of MAP would not have been so greatly impacted by the unequal

splitting of the milk sample, as these would likely still be positive for *MAP* in both fractions tested. Due to the finding of 'poor' agreement between results of the PMS-phage assay and PMS-culture, it was decided that it may be more appropriate to combine the test results when assessing diagnostic potential of the PMS-based assays. Diagnostic sensitivity (DSe) increased from 0.250 (PMS-culture) and 0.325 (PMS-phage assay) to 0.525 when results of the two PMS-based tests were combined. DSp of the combined PMS-based methods was the same as PMS-culture (0.962), which was lower than for the PMS-phage assay (1.000).

The DSp and DSe of the PMS-phage assay, PMS-culture, and the combined PMS-369 based assays, were determined relative to the MAP infection status of the animals supplying 370 the milk samples (Table 2). There was a two year gap between faecal culture and serum-371 ELISA testing of the animals in 2013, upon which each animal's infection status was 372 assigned, and the collection of milk samples for PMS-phage assay and PMS-culture testing 373 in December 2015 and January 2016. This situation was not ideal, but was unavoidable 374 given that regular Johne's testing of cattle in these Northern Ireland dairy herds was not 375 376 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 similar to that estimated for faecal culture (0.20) of Irish cattle by More et al. (2013); DSe of 380 381 the PMS-phage assay and combined PMS-based tests were higher (0.325 and 0.525, respectively). The infection status of the animals in negative Herd C could have changed 382 between 2013 and 2015/16. However, according to the animal health database of the 383 Department of Agriculture, Environment and Rural Affairs for Northern Ireland, Herd C is still 384 officially recorded as MAP 'non-infected' (Dr Lyanne McCallan, Agri-Food and Biosciences 385 Institute for Northern Ireland, personal communication). PMS-culture results suggest there 386 were four cows in negative Herd C that may have been MAP-infected at the time of milk 387 collection in 2015/16. Since these milk samples were PMS-culture positive, and not just 388 389 PMS-phage assay positive, it seems rather unlikely they could be false positive results. The detection of viable *MAP* in some milk samples from supposedly 'non-infected' animals reduced the DSp estimate from 1.000 for the PMS-phage assay to 0.962 for PMS-culture and for the combined use of the PMS-based tests (Table 2).

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

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

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

^{*} 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.

+ Milk samples that subsequently tested PMS-culture positive, one of which had a negative

⁵⁰⁷ PMS-phage assay result (Herd A, sample 10).

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

