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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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24 **ABSTRACT**

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

28 **Methods and Results:** Inclusivity, specificity and limit of detection 50% (LOD₅₀) of
29 the optimised PMS-phage assay were assessed. Plaques were obtained for all 43
30 MAP strains tested. Of 12 other *Mycobacterium* spp. tested, only *M. bovis* BCG
31 produced small numbers of plaques. LOD₅₀ of the PMS-phage assay was 0.93 MAP
32 cells per 50 ml milk, which was better than both PMS-qPCR and PMS-culture. When
33 individual milks (n=146) and bulk tank milk (BTM, n=22) obtained from Johne's
34 affected herds were tested by the PMS-phage assay, viable MAP were detected in
35 31 (21.2%) of 146 individual milks and 13 (59.1%) of 22 BTM, with MAP numbers
36 detected ranging from 6-948 PFU per 50 ml milk. PMS-qPCR and PMS-MGIT culture
37 proved to be less sensitive tests than the PMS-phage assay.

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

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

46

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

49 **Introduction**

50

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

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

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

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

92 We recently combined an optimized phage amplification assay (Foddai *et al.*
93 2009) with selective peptide-mediated magnetic separation (PMS) (Foddai *et al.*
94 2010b) to achieve a rapid novel detection test for viable MAP. The PMS-phage
95 method exploits the use of D29 mycobacteriophage and is able to provide rapid
96 enumeration of viable MAP in milk and veterinary samples within 48 h (Foddai *et al.*
97 2011), based on the number of plaques (lysis areas) produced on a lawn of fast-
98 growing *M. smegmatis*. More recently, an optimized milk sample preparation protocol

99 to maximize accuracy of MAP counts when testing cows' milk was described (Foddai
100 and Grant 2015). The aims of the present study were: (1) to assess inclusivity and
101 specificity of the recently optimised PMS-phage assay by testing a large number of
102 MAP strains and other bacterial milk isolates; (2) to compare the limit of detection
103 50% (LOD₅₀) of the optimised PMS-phage assay with those of PMS-culture and
104 PMS-qPCR targeting both IS900 and f57 by testing artificially contaminated milk
105 samples; and (3) to test individual raw milk samples and bulk tank milk from dairy
106 herds affected by JD to assess the applicability of the optimised PMS-phage assay
107 for raw milk testing.

108

109 **Materials and methods**

110

111 **Bacterial strains and growth conditions**

112 Forty-three MAP strains (three type strains and 40 cattle, raw and pasteurised cow's
113 milk, untreated water, or Crohn's disease isolates, acquired or isolated over the past
114 20 years and available within the Queen's University Belfast (QUB) culture
115 collection), 12 other *Mycobacterium* spp. (Table 1), and five bacterial isolates (one
116 Gram positive coccus and four Gram positive rods) obtained by plating a sample of
117 raw cows' milk on Nutrient agar (Oxoid Ltd., Basingstoke, UK), were used in this
118 study. All MAP strains were grown in a shaker incubator for 4-6 weeks at 37°C to
119 stationary phase in Middlebrook 7H9 broth containing 10% (v/v) OADC supplement
120 (both from Difco) and 2 µg per ml mycobactin J (Synbiotics Europe SAS, Lyon,
121 France). All *Mycobacterium* spp. were cultivated at appropriate temperatures
122 (dependent on species) to stationary phase (between 3 and 15 days) in the same

123 7H9 medium without the addition of mycobactin J. Milk isolates were grown
124 overnight in 10 ml of nutrient broth at 25°C.

125

126 **Peptide-mediated magnetic separation (PMS)**

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

135

136 **Optimised phage amplification assay**

137 The optimised phage assay was carried out as previously described by Foddai *et al.*
138 (2009). Briefly, after overnight incubation of samples at 37°C in 1 ml of 7H9 medium
139 containing 2 mmol l⁻¹ CaCl₂, samples were incubated for 2 hours at 37°C with 10⁸
140 D29 mycobacteriophage before treatment with 100 mmol l⁻¹ ferrous ammonium
141 sulphate (FAS, Sigma) for 10 min at room temperature to inactivate any
142 exogenous/non-adsorbed seed phage. Samples were then mixed with 5 ml 7H9
143 medium containing 2 mmol l⁻¹ CaCl₂ and returned to the incubator at 37°C for a
144 further 90 min before being plated with tempered 7H9 agar and 1 ml *Mycobacterium*
145 *smegmatis* mc² 155 (10⁸ CFU per ml). Plaques were counted following overnight
146 incubation of plates at 37°C.

147

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

150 Before being tested by the optimised PMS-phage assay, all stationary MAP broth
151 cultures were declumped by ultrasonication applied as previously described (Foddai
152 and Grant 2015) at 37 kHz for 4 min on ice in a Ultrasonic PH 30 (Fisher Scientific
153 Ltd) and then tested for purity (presence of only red acid-fast cells) by Ziehl–Neelsen
154 (ZN) staining. The number of cells per ml of broth was estimated by measuring the
155 optical density at 600nm (OD₆₀₀) using a WPA CO8000 cell density meter (SISLAB,
156 Italy). For each sample, optical density was adjusted to an OD₆₀₀ of 0.1
157 (approximately 10⁶-10⁷ MAP cells per ml) followed by serial dilution of cultures in
158 PBS-T20. PBS-T20 suspensions containing approximately 10²-10³ MAP per ml were
159 finally processed through optimised PMS-phage assay to assess inclusivity of the
160 assay. The number of MAP cells detected was indicated by plaques (zones of
161 clearing) produced on agar plates containing 5 ml of molten 7H9 agar and 1 ml of *M.*
162 *smegmatis* mc² 155, and reported as plaque-forming-units (PFU) per ml. The
163 experiment was repeated twice for each of the 43 MAP strains.

164

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

167 Broth suspensions of 12 environmental *Mycobacterium* spp. and five raw milk
168 isolates spiked at three levels (10-10², 10²-10³, 10³-10⁴ cells per ml) were processed
169 through the phage assay to assess specificity of the assay for MAP. For each
170 sample tested, inoculum was prepared as described above. Number of bacterial
171 cells per ml stationary phase broth was estimated by measuring and adjusting the

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

174

175 **Comparison of the limit of detection 50% (LOD₅₀) of the PMS-phage assay,**
176 **PMS-culture and PMS-qPCR**

177 Ultra-high temperature (UHT) milk purchased from a local supermarket and
178 artificially contaminated at different levels with MAP was used to compare the LOD₅₀
179 of the three detection methods. Three sets of 50 ml UHT milk samples were spiked
180 in triplicate at four levels of MAP contamination (target final concentrations 10²-10³,
181 10-10², 1-10 and 0 PFU per 50 ml) by adding 1 ml of an appropriate dilution of MAP
182 NCTC 8578 or ATCC 19698 per 50 ml of milk. The number of MAP added at the
183 highest spiking level, in each case, was determined by the optimised phage
184 amplification assay (no PMS) applied to the diluted MAP culture used as inoculum.
185 Each set of 50 ml milk samples was processed through PMS followed by either the
186 phage amplification assay, culture or qPCR. Irrespective of detection method, milk
187 sample preparation included: (i) centrifugation at 2,500 x g for 15 min, (ii) declumping
188 by ultrasonication applied to the resuspended pellet fraction (Foddai and Grant,
189 2015), (iii) PMS, and then one of the following detection methods:

190 a) Phage assay applied after a previous overnight incubation of bead samples in
191 7H9 Middlebrook broth supplemented with 10% (v/v) OADC, 2 mmol l⁻¹ CaCl₂ and
192 NOA Antimicrobial Supplement (Product code 3A201N-300, Abtek Biological Ltd,
193 Liverpool UK; final concentrations per ml of broth: Nystatin 50 IU, Oxacillin 2 µg,
194 Aztreonam 30 µg);

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

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

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

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

204

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

206 A total of 146 individual raw milk samples and three bulk tank milks (BTM) sourced
207 from a large JD affected dairy herd in southern England, identified with the help of Mr
208 Peter Orpin (Park Veterinary Group, Leicester), and 19 BTM obtained from dairy
209 herds in Scotland with the help of Dr George Caldow (SAC Consulting Services, St
210 Boswells) were tested for MAP. The individual milk samples were collected by farm
211 workers during one morning milking session into sterile 50 ml centrifuge tubes
212 supplied by QUB. Milk samples were immediately frozen and stored overnight on
213 farm before being transported to QUB in insulated boxes by an overnight courier.
214 Samples arrived at QUB in a partially frozen state and were immediately placed in -
215 80°C freezer. Before application of the PMS-phage assay sample preparation
216 included: thawing overnight in the refrigerator at 4°C, room temperature for 1 h
217 before centrifugation of 50 ml milk at 2500 x g for 15 min at room temperature,
218 resuspension of milk pellet fraction in 1 ml PBS-T20, application of ultrasonication to
219 disperse MAP clumps (Foddai and Grant 2015), and then PMS. After PMS, samples
220 were concentrated in a final volume of 150 µl of sterile RNase/DNase free water
221 (Sigma) and divided into three aliquots (50 µl) to be processed via the three different

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

224

225 **Phage amplification assay and confirmatory Plaque PCR**

226 Following overnight incubation of the bead samples at 37°C in a final volume of 1 ml
227 7H9 medium supplemented with 10% (v/v) OADC, 2 mmol l⁻¹ CaCl₂ and NOA
228 Antimicrobial Supplement (as described above), the phage amplification assay was
229 carried out as described by Foddai *et al.* (2009). After overnight incubation, plaques
230 (1 to, maximum, 10 depending on PFU counts) from positive phage assay plates
231 were harvested and processed through DNA extraction as described by Swift *et al.*
232 (2013). Plaque DNA in each aliquot was concentrated and purified through
233 Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, Ca, USA), and then
234 subjected to IS900 Plaque-PCR as described by Stanley *et al.* (2007).

235

236 **Culture and confirmation of MAP isolation**

237 After PMS, samples were cultivated in BD BBL MGIT™ Mycobacteria Growth
238 Indicator Tubes containing 4 ml of modified Middlebrook liquid broth enriched with
239 0.5 ml BBL™ MGIT™ OADC and 0.1 ml MGIT™ PANTA™ antibiotic mixture (all
240 Becton Dickinson Limited, USA). Cultures were incubated for 16-20 weeks at 37°C
241 and were periodically monitored using a BACTEC MicroMGIT reader (Becton
242 Dickinson Limited, USA). Liquid broths showing evidence of growth were tested by
243 ZN staining for the presence acid-fast bacilli and by IS900 PCR (Naser *et al.* 2013)
244 to identify MAP positive cultures. All cultures that tested acid-fast and IS900 PCR
245 positive were then subjected to a mild chemical decontamination treatment in 0.75%
246 (w/v) hexadecylpyridinium chloride (HPC) for 60 min at room temperature before

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

250

251 **Real time qPCR**

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

263

264 **Statistical analysis of results**

265 For inclusivity and specificity testing, positive or negative results for the plaque assay
266 applied after PMS were used as a final result. Limit of detection (LOD₅₀) and
267 associated 95% confidence limits of the three methods assays (PMS-phage assay,
268 PMS-culture, PMS-IS900 and f57 qPCR) were estimated using the generalized
269 Spearman-Kärber LOD₅₀ calculation for 4-level spiking protocols (AOAC
270 International 2006). Kappa agreement between test results was determined using
271 EpiTools Epidemiological Calculators (<http://epitools.ausvet.com.au>).

272

273 **Results**

274

275 **Confirmation of inclusivity of the novel PMS-phage assay**

276 All 43 MAP strains tested by the PMS-phage assay yielded plaques, confirming
277 100% inclusivity of the test (Figure 1). PFU counts observed for most of the MAP
278 strains (40 of 43, 93%) ranged from 100 to 300 (mean 142.26 ± 51.97 PFU per ml);
279 the number of plaques expected based on the culture dilution tested. Three MAP
280 strains (NCTC 8578, 307R and Van Veen 52991-1) showed greater variability in
281 counts between replicates and a higher mean PFU ml⁻¹ count (mean 681.50 ± 165.91
282 PFU per ml) than was observed for the other strains tested (Figure 1).

283

284 **Confirmation of specificity of the PMS-phage assay**

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

292

293 **Comparison of the LOD₅₀ of the PMS-phage assay, PMS-culture and PMS- 294 qPCR**

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

297 MAP NCTC 8578; determined by the optimised phage amplification assay (without
298 PMS). Higher detection sensitivity for MAP was exhibited by the PMS-phage assay
299 compared to the PMS-qPCR and PMS-culture assays applied in parallel to spiked
300 milk samples. The LOD₅₀ of the PMS-phage assay was estimated to be 0.90 and
301 0.95 PFU per 50 ml milk (calculation of 95% CI not possible) for MAP strains NCTC
302 8578 and ATCC 19698, respectively (Table 2). PMS-IS900 qPCR had the next
303 highest detection sensitivity, followed by PMS-f57 qPCR, and finally PMS- culture.
304 The LOD₅₀ of PMS-IS900 qPCR was 136.7 (95% CI: 21.4-872.9) and 134.55 (95%
305 CI: 23.45-772.80) MAP cells per 50 ml milk for strains NCTC 8578 and ATCC 19698,
306 respectively; the LOD₅₀ of f57 qPCR was 303.7 and 291.00 MAP cells per 50 ml milk
307 (calcalaton of 95% CI not possible) for strains NCTC 8578 and MAP ATCC19698,
308 respectively (Table 2). An LOD₅₀ for PMS-culture could not be calculated because
309 none of the milk samples spiked at the highest level (10²-10³ CFU per 50 ml) yielded
310 colonies on HEYM agar plates, and thus use of the Excel LOD₅₀ calculator (AOAC
311 International, 2006) was not valid.

312

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

314 Table 3 summarises the number and percentage of individual and bulk tank milk
315 samples testing MAP positive by the three detection methods. Overall, 59 milk
316 samples yielded plaques in the PMS-phage assay, some or all of which (depending
317 on numbers present) were harvested and subjected to IS900 Plaque-PCR. Of the
318 59 plaque composites tested by IS900 Plaque-PCR, 44 (31 from individual milks and
319 13 from BTM, 74.6%) yielded clear, positive IS900 Plaque-PCR results, 5 (8.5%)
320 yielded inconclusive PCR results, 10 (16.9%) yielded negative PCR results, and one
321 was not tested because plaques had merged and individual plaques could not be

322 harvested. Therefore, viable MAP were confirmed to be present in 31 (21.2 %) of
323 146 individual milks and 13 (59.1%) of 22 BTM tested by the PMS-phage assay
324 (Table 3). Mean viable MAP counts indicated by the PMS-phage assay in the
325 positive individual raw milks and bulk tank milks were 228.1 PFU per 50 ml (range 6-
326 948 PFU per 50 ml) and 136.83 PFU per 50 ml (range 18-685 PFU per 50 ml),
327 respectively.

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

340 When cultured in MGIT broth (not HEYM agar plates, as in first part of the
341 study) after PMS, 49 (33.6%) of the 146 individual raw milk cultures and 16 (72.7%)
342 of the 22 BTM cultures showed evidence of acid-fast bacteria when tested by ZN
343 staining; many of these cultures were mixed cultures and showed evidence of the
344 presence of non-acid-fast bacteria. Isolation of MAP cells was confirmed in 17
345 (11.6%) individual raw milk and 11 (50%) BTM cultures by IS900 PCR (Naser *et al.*
346 2013). Inter-relationships between results of the PMS-phage assay, PMS-culture

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

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

367

368 **Discussion**

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

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

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

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

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

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

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

449 Detection sensitivity, specifically LOD₅₀, was the last aspect of the PMS-
450 phage assay investigated in the present study. Evidence to date suggests that MAP
451 is typically present in raw milk in low numbers (Sweeney *et al.* 1992; Slana *et al.*
452 2009). Since conventional culture often does not have sufficient sensitivity (Slana *et*
453 *al.* 2008a), an alternative detection method is required to rapidly demonstrate the
454 presence of viable MAP in milk and achieve more accurate enumeration. Results
455 from testing of both artificially and naturally infected milk samples indicate that the
456 optimised PMS-phage assay possesses the sensitivity needed to detect low levels of
457 MAP in raw milk. The optimised PMS-phage assay demonstrated higher sensitivity
458 than both PMS-culture (on HEYM agar plates) and PMS-qPCR, targeting either
459 IS900 or f57, when the test was used to test whole milk spiked at different levels with
460 two MAP strains. Plaques were observed from all the milk samples, including those
461 originally spiked with 1-10 MAP cells per 50 ml. Higher LOD₅₀ were observed for the
462 two other PMS-based detection methods (≥ 100 PFU per 50 ml). The higher MAP
463 detection rates observed with the PMS-phage assay during raw milk testing further
464 confirmed the higher sensitivity of this test compared to PMS-culture and PMS-
465 qPCR. Viable MAP cells were detected by PMS-phage assay in 21.2% of individual
466 milk samples and 59.1% of BTM samples tested. These detection rates were
467 consistently higher than corresponding rates observed for PMS-IS900 qPCR (9.1%
468 and 45.4%, respectively), PMS-f57qPCR (2.6% and 9.1%, respectively) and PMS-
469 culture (11.6% and 50%, respectively). It should be noted that for raw milk testing
470 MGIT broth was employed rather than HEYM agar plates for culture after PMS, and
471 it was noticeable that the detection sensitivity of PMS-culture was much improved,

472 compared to the outcomes of LOD₅₀ determinations using spiked milk. From past
473 experience, liquid culture does tend to be more conducive for MAP growth than solid
474 culture. The optimised PMS-phage assay proved to be a more sensitive and quicker
475 alternative to conventional culture for demonstrating the presence of viable MAP in
476 cows' milk.

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

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

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

504 To conclude, this study has demonstrated that the optimised PMS-phage
505 assay is 100% inclusive for all MAP strains tested, >99% specific for MAP, and has a
506 LOD₅₀ of ~1 PFU per 50 ml milk. When used in combination with the optimized milk
507 sample preparation protocol (Foddai and Grant, 2015), the assay detected viable
508 MAP in raw milk from a substantial proportion (21.2%) of individual cows in a JD
509 affected dairy herd (mean MAP count 228.1 PFU per 50 ml) and in 59.1% of BTM
510 from JD affected dairy farms in Scotland (mean MAP count 136.83 PFU per 50 ml).
511 This study has also demonstrated that the optimised PMS-phage assay is more
512 sensitive than both PMS-qPCR and PMS-culture (on HEYM or in MGIT liquid
513 medium). Unfortunately, in its present multi-step format, the PMS-phage assay does
514 not represent a test that could be easily adopted by the dairy industry for routinely
515 screening large numbers of milk samples to detect the presence of MAP. Efforts are
516 continuing to streamline the assay, whilst maintaining its excellent detection
517 sensitivity, to make it suitable for that purpose.

518

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525

526 **Conflict of interest**

527 No conflict of interest declared.

528

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

| Test <i>Mycobacterium</i> sp. | Strain ID or Source |
|-------------------------------------|----------------------------------|
| <i>M. avium</i> subsp. <i>avium</i> | AFBI ^a |
| <i>M. avium</i> subsp. <i>avium</i> | NCTC ^b 13034 |
| <i>M. bovis</i> BCG | NCTC 5692 |
| <i>M. fortuitum</i> | NCTC 10394 |
| <i>M. goodii</i> | NCTC 10267 |
| <i>M. intracellulare</i> | NCTC 10425 |
| <i>M. kansasii</i> | NCTC 10268 |
| <i>M. marinum</i> | AFBI |
| <i>M. scrofulaceum</i> | AFBI |
| <i>M. smegmatis</i> | mc ² 155 ^c |
| <i>M. terrae</i> | AFBI |
| <i>M. xenopi</i> | AFBI |

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

649 ^b Purchased from National Collection of Type Cultures, Colindale, London.

650 ^c Originally provided by Dr Ruth McNerney, London School of Hygiene and Tropical
 651 Medicine.

652

653

654 .

655 **Table 2.** Comparison of limits of detection 50% (LOD₅₀) of the different PMS-based assays. Data represent number of samples
 656 test positive of total number of samples tested at each spiking level.

657

| 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 | - † |
| 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 | - |

658 * LOD₅₀ could not be calculated because no spiking level yielded a partially positive response, i.e. <3/3

659 † LOD₅₀ could not be calculated because no milk sample at highest spiking level tested positive after PMS-culture.

660

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

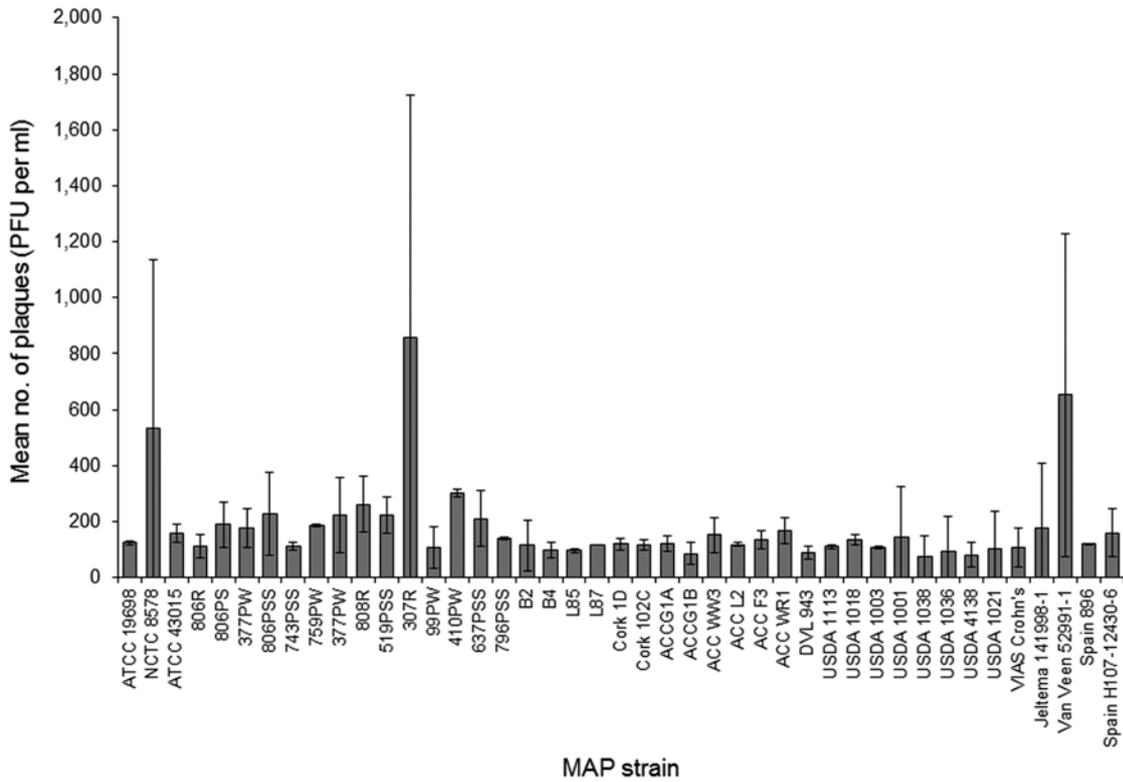
| Type of milk sample | PMS-phage assay* | | | PMS-qPCR | | | | | | PMS-MGIT culture † | | |
|---------------------|------------------|---------|-------|-------------|---------|-------|------------|---------|-------|--------------------|-----------|-------|
| | No. tested | No. Pos | % Pos | IS900 | | | F57 | | | 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 |

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

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

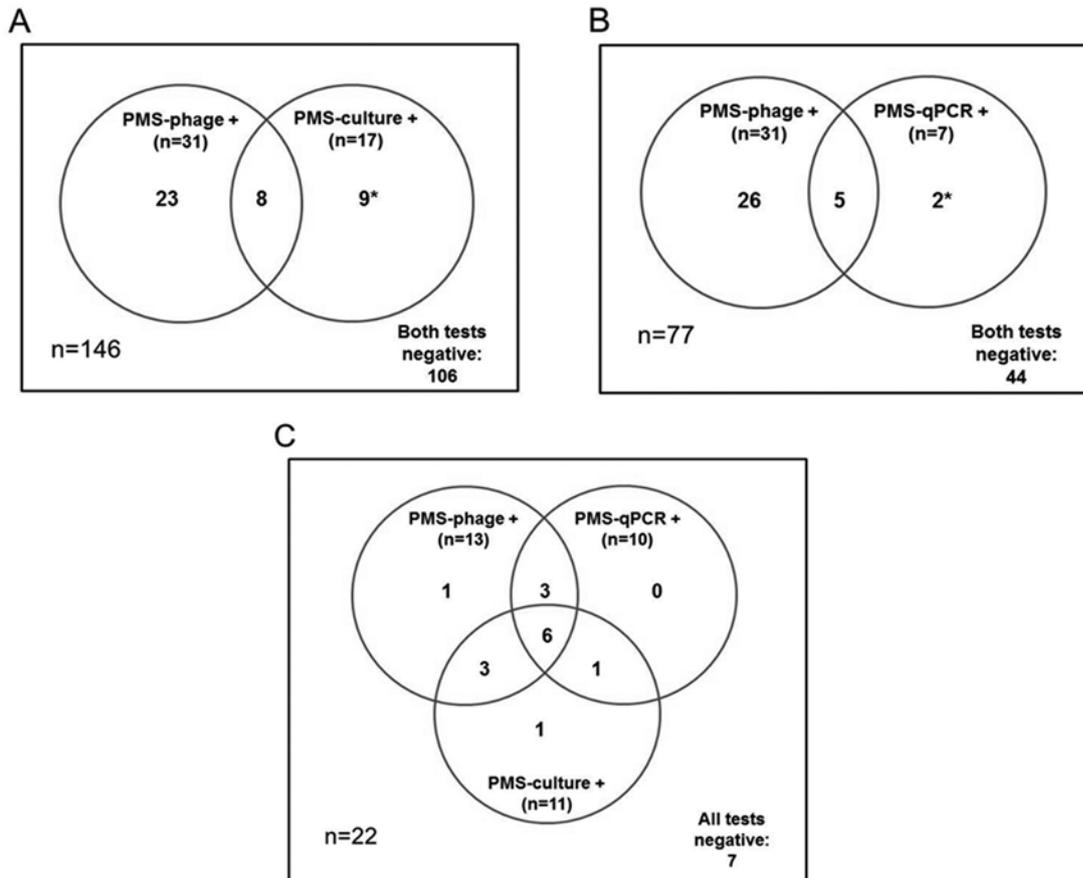
666 ‡ All 146 individual milk samples were not tested by either PMS-qPCR assay due to cost constraints. The 77 samples tested
 667 included 47 samples that had yielded plaques with the PMS-phage assay plus 30 other randomly selected PMS-phage assay
 668 negative samples.

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



672

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



678