Use of untargeted magnetic beads to capture *Mycobacterium smegmatis* and *Mycobacterium avium paratuberculosis* prior detection by mycobacteriophage D29 and Real-Time-PCR

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23 **ABSTRACT**

24 Untargeted magnetic beads were evaluated to capture *Mycobacterium smegmatis* and *Mycobacterium*
25 *avium* subspecies *paratuberculosis* (MAP) from spiked feces, milk, and urine. Untargeted magnetic
26 beads recovered more *M. smegmatis* cells from PBS suspension than the typical centrifugation method;
27 96.31% of 1.68 x 10^4 CFU/mL viable *M. smegmatis* were recovered by beads and 0% by centrifugation.
28 Likewise, the *F57*-qPCR detection of MAP cells was different whether they were recovered by beads or
29 centrifugation; cycle threshold (Ct) was lower ($p<0.05$) for the detection of MAP cells recovered by
30 beads than centrifugation, indicative of higher sensitivity. Magnetic separation of MAP cells from milk,
31 urine, and feces specimens were detected by *F57* and *IS900* sequences. Ct values demonstrated that
32 beads captured no less than 10^9 CFU/mL from feces and no less than 10^4 CFU/mL from milk and urine
33 suspensions. In another detection strategy, *M. smegmatis* coupled to magnetic beads were infected by
34 mycobacteriophage D29. Plaque forming units were observed after 24 h of incubation from urine
35 samples containing 2 x 10^5 and 2 x 10^3 CFU/mL *M. smegmatis*. The results of this study provide a
36 promising tool for diagnosis of Johne's disease and other mycobacterial diseases such as tuberculosis.

37 **1. Introduction**

38

39 The genus *Mycobacterium* is grouped in *Mycobacterium tuberculosis* complex (MTBC) and non-
40 tuberculosis mycobacteria (NTM) (1) and can cause multiple diseases in humans and animals (2,3). *M.*
41 *tuberculosis* (MTB), the etiological agent of tuberculosis (TB) in humans causes millions of deaths every
42 year, mainly in developing countries (4), can be isolated from sputum (5), lavage fluid (6,7), gastric
43 lavage (8,9), urine (10–12), and stools (13,14). *M. avium* subspecies *paratuberculosis* (MAP), a NTM that
44 cause Johne's disease (JD) or paratuberculosis, has been isolated from intestinal samples of domestic
45 animals (e.g., cattle, sheep, goats, and farmed deer), wildlife (e.g., rabbit, hare, fox, stoat, weasel, crow,
46 rook); and human beings (15)(16)(17).

47 Definitive diagnostic of TB and JD is based on the isolation of MTB and MAP from human and
48 animal's specimens on culture media. For that, the specimen, which can be contaminated with normal
49 flora organisms, should be decontaminated and centrifuged (18). Classical decontamination methods to
50 isolate MTB and MAP using the culture method are based on the use of *N*-acetyl-L-cysteine–sodium
51 hydroxide (NALC-NaOH) (18) and hexadecylpyridinium chloride (HPC) (19), respectively for MTB and
52 MAP, and incorporate the concentration of the specimen by centrifugation at 3,000 *x g* at 4°C for 15-20
53 min (18–20). Given that these chemical decontaminants can kill a portion of mycobacteria present in the
54 specimen (19–22), and that the availability of refrigerated centrifuges with sealed safety cups and
55 aerosol-tight rotors may be challenging in laboratories from low- and middle-income countries (23),
56 additional efforts should be focused on the development of new and simple methods that enable the
57 separation of MTB and MAP from specimens without affecting their viability to grow on culture media or
58 be detected.

59 Magnetic-separation methods, already established as routine methods to detect *Listeria*
60 *monocytogenes* (24), *Salmonella* spp. (25), and *Escherichia coli* O157:H7 (26) (27) in food and veterinary
61 clinical samples, are promising alternatives to selectively separate and concentrate MTB (28–32) and
62 MAP (33,34). Magnetic bead characteristics (composition, size, concentration, and surface modification)
63 and the nature of coating ligands (antibodies, peptides, chemicals) are important factors enabling the
64 concentration of the target bacteria and increasing the performance of further complementary
65 detection methods such as culture, PCR, microscopy, antigen detection immunoassay, or phage assay
66 (33,35,36).

67 Tosyl-activated magnetic beads (e.g., Dynabeads M-280 Tosylactivated) can bind covalently to
68 primary amine and sulfhydryl groups in proteins and peptides (37). Importantly, some surface proteins

69 have been identified on mycobacteria (38–40). As per their characteristics, we postulate that Tosyl-
70 activated magnetic beads may interact with these surface proteins enabling the capture of *M.*
71 *smegmatis* and MAP cells in suspension. Because the use of untargeted beads to capture MAP cells
72 would diminish laboratory work and costs, and would increase affordability in countries with a high
73 burden of TB and JD, this study determined the potential of Tosyl-activated Dynabeads M-280
74 (untargeted magnetic beads) to capture *M. smegmatis* and MAP from spiked urine, feces, and milk
75 samples prior to detection by real-time PCR (qPCR) and phage D29.
76
77 **2. Material and Methods**
78
79 **2.1. Bacterial cultures**
80 MAP ATCC 19698 was cultured in Middlebrook 7H9 broth (BD Biosciences, San Jose, CA, USA)
81 supplemented with an oleic acid–albumin–dextrose–catalase mixture (BD Biosciences) and mycobactin J
82 (Allied Monitor, Fayette, MO, USA) at 37°C with constant shaking at 200 rpm for 60 d. *M. smegmatis*
83 mc2155 cells were cultured under the same conditions but without mycobactin and for 7 d. *Escherichia*
84 *coli* BL21 (DE3) was cultured in Luria–Bertani (LB) broth at 37°C for 24 h with shaking. Stock *M.*
85 *smegmatis* and *E. coli* cells suspensions were adjusted to an optical density at 600 nm (OD_{600}; measured
86 using a UV-visible spectrophotometer, Cytation 5, Biotek Inc.) measurement of 1. Then four serial 10-
87 fold dilutions of the stock suspension were made for CFU counting on LB agar plates.
88 **2.2. Magnetic beads assay**
89 Tosyl-activated Dynabeads™ M-280 (Invitrogen)(37) were used for cell capture. Briefly, 21 µl of
90 beads was transferred to one sterile microcentrifuge tube of 2-ml capacity (microtube) and washed
91 twice with 1 ml of PBS (pH 7.4) for 1 min each time. The microtube was placed on a magnet (Magna grip
92 rack, Millipore) for removing supernatant. Washed beads were then added to microtubes containing 1
93 mL of PBS, milk, urine, and feces suspensions spiked with various levels of *M. smegmatis* or MAP cells.
94 These tubes were placed in a microtube rotator (Thermo Scientific™ Labquake™, Edmonton, AB,
95 Canada) at room temperature for 1 h. The microtubes were then placed on a magnetic rack for 1 min to
96 carefully remove the supernatant to another microtube. Magnetic beads coupled to *M. smegmatis* or
97 MAP were washed twice with 1 mL PBS for 1 min before moving on to the detection assays using qPCR
98 or phage D29.
99 **2.3. Recovery of viable M. smegmatis and E. coli cells by magnetic beads**

100 ***M. smegmatis.*** Suspensions of *M. smegmatis* cells were prepared from a stock. It was adjusted to
101 OD_{600} ~1 (microtube#1), and then serial 10-fold dilutions in PBS were performed (microtube#2,
102 microtube#3, and microtube#4) to obtain different cell concentrations for assays. Determination of
103 viable *M. smegmatis* recovered by beads and centrifugation was determined based on the number of
104 cells isolated from the supernatant, as sedimenting cells may form clumps which can lead to an
105 underestimation of *M. smegmatis* cells isolated on LB plates. To ensure all cells remained in the entire
106 volume of supernatant, the experiments were optimized for 2-mL microtubes. Two aliquots (1 mL) of
107 each microtube were transferred to two microtubes for centrifugation (control) and beads methods.
108 Microtube#1 (control) was centrifuged at 3,000 *x g* at 4°C for 16 min, and the total volume of
109 supernatant was cultured on LB agar plates for counting cells that were not sedimented. Another
110 microtube#1 was assayed for magnetic capture, and the total volume of supernatant was carefully
111 removed and transferred to one plastic cuvette for measuring turbidity via OD_{600} and for colony counts
112 on LB-agar plates. The same procedure, except measuring turbidity, was applied for microtube#2 and
113 microtube#3. From microtube#4, *M. smegmatis* cells recovered by beads and centrifugation were
114 resuspended in 1 mL PBS, vortexed vigorously and plated on LB agar plates. Likewise, the total
115 supernatant volume was plated on LB agar for CFU count. The experiment was repeated 3 times.
116 ***E. coli.*** From a stock of *E. coli* suspension (adjusted to OD_{600} ~1), 2 aliquots (1 mL each) were transferred
117 to 2 microtubes to evaluate the concentration of cells by centrifugation (2,000 *x g*, 4°C, 16 min) and
118 capture by magnetic beads. Recovery of cells by centrifugation (control) was done following the
119 procedure described for the microtube#1 with *M. smegmatis*. The experiment was repeated 3 times.

120 **2.4. MAP cells DNA extraction**

121 DNA extraction was performed using a GenoLyse® kit (Hain Lifescience, Nehren, BW, Germany)
122 as per manufacturer's instructions with some modifications. Briefly, MAP cells concentrated by
123 centrifugation or magnetic beads were resuspended in 100 µl of A-Lys, vortexed, centrifuged at 10,000 *x*
124 *g* for 1 min, and incubated for 5 min at 95°C in a water bath. Then, 100 µl of A-NB buffer was added to
125 the lysate, vortexed, and centrifuged at 10,000 *x g* for 5 min. Supernatant was transferred to sterile 1.5-
126 ml microtubes. Concentration of isolated DNA was determined by measuring sample absorbance at 260
127 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).
128

129 **2.5. Quantitative qPCR assay**

130 This procedure was done as per Singh et al. (36). Briefly, qPCR amplification targeting *IS900* and
131 *F57* genes was done using Taqman specific probes and StepOnePlus Real Time PCR System (Applied

132 Biosystems). The 10-µL PCR mixture comprised 5 µL of TaqMan® Universal Master Mix II (Applied
133 Biosystems) with uracil-N-glycosylase (UNG), 200 nM of each primer and 250 nM of fluorogenic probe
134 specific to *IS900* and *F57*; the sequence of the primers and probes used were described elsewhere (36).
135 For each template DNA sample, 4 µL was added in the reaction mixture. qPCR conditions were as
136 follows: 50°C for 2 min for UNG treatment, initial denaturation at 95°C for 10 min, 40 cycles at 95°C for
137 15 sec, and at 60°C for 1 min. Each qPCR amplification targeting *IS900* and *F57* genes was run in
138 triplicate with negative and positive controls. Obtained data were analyzed in the form of threshold
139 cycle (Ct) values.
140
141 **2.6. Detection of MAP cells by qPCR**
142 This experiment was done to evaluate the recovery of MAP cells by beads and centrifugation
143 through the detection of DNA of the total, viable and non-viable, cells sedimented. Efficiency of beads
144 and centrifugation to capture and sediment cells, respectively, was determined by using two referential
145 MAP concentrations (5 x 10^7 CFU/ml and 5 x 10^5 CFU/mL). Briefly, two serial 10-fold dilutions were
146 prepared from the stock (5 x 10^7 CFU/mL) of MAP suspension in PBS. Two aliquots (1 mL) of the stock
147 were transferred to two microtubes to evaluate the recovery of MAP by centrifugation (control) and
148 magnetic beads. MAP cells recovered by beads and centrifugation (3,000 *x g*, 4°C, 16 min) were analyzed
149 by *F57* qPCR. The same procedure was performed for the second serial dilution containing 5 x 10^5
150 CFU/mL. Each condition was repeated three times.
151
152 **2.7. Capture of MAP in milk, urine and feces by beads**
153 **From milk and urine suspension.** Three sterile microtubes containing 0.9 mL of PBS (pH 7.4), 0.9 mL of
154 urine from an apparently healthy human or 0.9 ml of skim milk (Dairyland®) were spiked with 0.1 mL of
155 10^8 CFU/mL MAP cells. Proteinase K (Pk) was added to the microtube containing milk and incubated at
156 70°C for 10 min before performing the magnetic beads assay. MAP cells recovered using beads were
157 analyzed by qPCR. The same procedure was applied to other microtubes containing fewer MAP cells
158 (10^6, 10^5, 10^4 CFU/mL). The experiment was run in triplicate and repeated twice.
159 **From feces suspension.** Cow feces of a known MAP-shedding calf (41) was provided by the University of
160 Calgary. Feces suspension was prepared by mixing 3 g of apparently healthy cattle feces and 6 ml of PBS
161 in a 15-ml centrifuge microtube. After keeping the microtube in an upright position for 20 min, 0.9 mL of
162 supernatant was transferred to another microtube and spiked with 0.1 mL of 10^8 CFU/mL MAP
163 suspended in PBS. One microtube with 0.9 mL PBS was spiked with 0.1 mL of 10^8 CFU/mL MAP. Both

164 microtubes were used for magnetic beads assays and analyzed by *F57* qPCR. The experiment was run in
165 triplicate and repeated twice.
166
167 **2.8. Quantification of MAP cells recovered by beads**
168 MAP cell counts were assessed using qPCR based upon the single copy fragment *F57* (42).
169 Briefly, DNA calibration curves were prepared for each experiment by preparing serial 10-fold dilutions
170 of DNA sample obtained from MAP cell (adjusted to OD_{600} ~1). The initial DNA concentration of the first
171 serial dilution was measured through optical absorbance at 260 nm using a NanoDrop ND 1000
172 spectrophotometer (NanoDrop Technologies Inc.). DNA molecule copy number (reported as equivalent
173 CFU/mL) was determined using the formula: $(\text{ng DNA} \times 6{,}023 \times 10^{23})/(4{,}829{,}781 \times 10^{9} \times 660)$(43). The
174 number of *F57* (equivalent CFU) detected in each MAP sediment recovered by beads and control was
175 determined by using the respective threshold cycle (Ct) values.
176 (36)
177 **2.9. Magnetic separation of M. smegmatis from urine for mycobacteriophage D29 infection**
178 The experimental procedure was adjusted as per infection of MTB by phage D29 (44) and
179 doubling time (3 to 4 hours) of *M. smegmatis* (45). Two urine aliquots of 1 ml containing 2.0×10^{5} and
180 2.0×10^{3} CFU/mL *M. smegmatis* were transferred to two sterile microtubes. *M. smegmatis* captured by
181 beads from urine were detected by phage D29 (44,46) with some modifications. Briefly, *M. smegmatis*
182 cells captured by beads were resuspended in 150 µL of LB with 1 mM $CaCl_2$ (LB-S) and infected with 50
183 µL of phage D29 stock at 10^{8} PFU/mL and 37°C. Extracellular phage D29 was eliminated with 0.03 M
184 ferrous ammonium sulfate (FAS) for 10 min; then 0.5 mL of LB-$CaCl_2$ was added to the microtube to stop
185 FAS virucide activity. The total volume suspension was mixed with 4 mL of sterile molten agar (0.8%) and
186 1 mL of 10^{8} CFU/mL *M. smegmatis* suspension before pouring onto Petri dishes with LB agar. Petri
187 dishes were incubated for 24 h at 37°C and the number of infection plaques was counted. The
188 experiment was repeated 3 times.
189
190 **2.10. Statistical analyses**
191 Number of *IS900* and *F57* gene copies, determined in triplicate per each sediment recovered by
192 u-TDbeads and control, were plotted and analyzed by the Kruskal-Wallis test (GraphPad Instat, version
193 8; GraphPad Prism, La Jolla, CA). *P*-values < 0.05 were considered statistically significant.
194
195 **3. Results**

196

197 **3.1. Capture of M. smegmatis and E. coli by magnetic beads**

198 Magnetic beads captured *M. smegmatis* cells in PBS with greater capture efficiency compared to
199 *E. coli* cells (P <0.05). This is evident by visual inspection of supernatant remaining in cuvettes after
200 capture by beads (Fig. 1). Cuvette B2 shows low remaining *M. smegmatis* cell contents, unlike cuvette
201 A2 which contained *E. coli*, indicating that beads efficiently removed cells from suspension (Fig. 1). The
202 number of viable *M. smegmatis* cells isolated from the supernatant of each suspension showed higher
203 recovery of viable cells by beads (> 90%) than centrifugation. *E. coli* cells were not recovered by beads;
204 approximately 94% of cells remained in the supernatant (Table 1).

205 The effect of the centrifugation and magnetic beads methods on the viability of *M. smegmatis*
206 was confirmed by assessing suspensions at 1.68 x 10^4 CFU/mL (Fig. 2). As per one experiment results, 17
207 CFU of *M. smegmatis* isolated from the supernatant and 337 CFU recovered from sedimented beads,
208 337 CFU compressed 1326 CFU of *M. smegmatis* as consequence of the formation of clumps during the
209 magnetic separation (Fig. 2). Thus, the magnetic beads recovered more viable *M. smegmatis* from the
210 suspension (96%) than the centrifugation (0%). An electron microscopy image of the capture of *M.*
211 *smegmatis* cells by beads is presented in Fig. 3.

212

213 **3.2. Detection of MAP cells by qPCR**

214 The initial concentration of MAP cells in the first and second PBS suspensions (2.93 x 10^9 and
215 1.43 x 10^7 equivalent CFU/mL, respectively) was estimated by detecting *F57* sequence from all MAP cells
216 collected by centrifugation, which is used to concentrate 95% mycobacteria (18). Using *F57*-qPCR, there
217 was a difference (p=0.04) in recovery of MAP cells between the magnetic beads and centrifugation
218 methods: from the first suspension 2.8 x 10^9 CFU/mL (Ct = 17.89) were recovered by magnetic beads
219 and 3.2 x 10^9 CFU/mL (Ct 17.68) by centrifugation, whereas 3.9 x 10^7 CFU/mL (Ct = 23.94) were
220 recovered by magnetic beads and 1.4 x 10^7 CFU/mL (Ct = 25.43) by centrifugation ((p<0.0001); Table 2).

221

222 **3.3. Milk, urine, and feces suspension.**

223 Ct values demonstrated that magnetic beads captured no less than 10^9 CFU/mL from feces and
224 no less than 10^4 CFU/mL of MAP cells from milk and urine suspensions (Fig. 4). From milk suspensions,
225 the recovery rates, confirmed by *F57*-qPCR, were 6.11% of 8.92 x 10^9 CFUL/mL, 5.96% of 6.25 x 10^7
226 CFU/mL, and 0.62% of 3.80 x 10^4 CFU/mL. Pk was incorporated in milk suspensions as preliminary
227 experiments showed that its activity increased the capture of MAP cells by beads (data not shown).

228 Recovery rates of MAP cells from urine suspensions using *F57*-qPCR was 30.93% of 8.92 x 10^9, 43.25% of
229 6.25 x 10^7, 44.56% of 6.11 x 10^6 CFU/mL, and 8.60% of 4.92 x 10^5 CFU/mL. *IS900*-qPCR, unlike the *F57*
230 method, was more sensitive to detect the lowest (10^4 CFU/mL) MAP cells concentration from milk and
231 urine (Fig. 4 and Table 3). From feces, 0.66% of 8.92 x 10^9 CFU/mL MAP cells were captured and
232 detected by beads and *F57*-qPCR (*IS900* was not done). MAP cells in feces suspensions at lower
233 concentrations were not detected.
234
235 **3.4. Magnetic separation of M. smegmatis from urine for mycobacteriophage D29 infection**
236 The combination of magnetic beads and phage D29 enabled the detection of *M. smegmatis*
237 spiked in urine within 24 h (Fig. 5). Although the number of plaques observed for samples from urine
238 spiked with 2 x 10^5 CFU/mL and 2 x 10^3 CFU/mL *M. smegmatis* was lower than the respective cell counts
239 (Table 4), the PFU detected from the samples at higher cell concentration clearly prove the detection of
240 *M. smegmatis* within 24 h.
241
242 **4. Discussion**
243
244 Untargeted Tosyl-activated Dynabeads™ M-280 recovered more viable *M. smegmatis* cells
245 (>90%) from spiked PBS suspension with 10^7, 10^6, 10^5 and 10^4 CFU/mL than the typical centrifuge-based
246 method. Capture efficiency was estimated by counting unbound *M. smegmatis* cells (in supernatant)
247 that were not captured nor sedimented by beads. Counting cells sedimented by beads, even after
248 resuspending them in 1 mL before plating (47), could underestimate the real number of cells recovered
249 by beads due to formation of cell clumps during the sedimentation process. With regards to cell
250 viability, untargeted magnetic beads did not affect the viability of *M. smegmatis,* supported by growth
251 on LB agar plate and infection by phage D29. On the other hand, centrifugation had a negative effect on
252 the viability of *M. smegmatis* cells, regardless of cell concentration. Moreover, qPCR detection of MAP
253 cells, after sedimentation by centrifugation and magnetic beads, indicates that the use of the latter
254 would be preferable to precipitate cells from PBS suspension. Although the centrifugation method
255 (3,000 *x g* at 4°C for 16 min) has been established as a referential method for the recovery of 95% of
256 cells from suspension (18,48), we agree with others (49–51) that centrifugation may not be an
257 appropriate method to concentrate specimens containing small numbers of microorganisms. The
258 inefficiency of the centrifuge method may be related to the physiology of the bacteria; low recovery of
259 MAP cells from a stationary culture is expected as the majority of cells have a lower buoyant density due

260 to the presence of lipid bodies (52). Likewise, as others have reported for other bacteria undergoing
261 centrifugation (53), the low number of *M. smegmatis* colonies growing on LB agar may be explained by
262 the damage of some bacterial cells, including DNA, during the centrifugation.

263 Since detection of MAP cells from milk improved after treating samples with Pk, we postulate
264 that Tosyl groups can be blocked by milk proteins which would, therefore, affect the capture of MAP
265 cells. The positive effect of Pk on capture of MAP from milk was not observed in urine nor feces,
266 perhaps, due to the higher protein concentration (1.87%) of milk (54) compared to urine (55) and feces.
267 With regards to the magnetic separation of captured MAP cells from urine and feces, we postulate that
268 the differences observed in qPCR sensitivity when detecting MAP cells in milk, urine and feces can be
269 explained by the number of copies of the targeted genes *F57* (1 per genome) (56,57) and *IS900* (14-20
270 per genome)(57,58). Urea amine group, and other inhibitors such as polysaccharides originated from
271 vegetable material in the diet (59), gut flora, bile salts, and glucolipids (60), may react with Tosyl groups
272 and could explain decreases in the capture of cells by beads. Likewise, these inhibitors could be
273 transferred with the beads coupled to MAP cells and reduce the amplification efficiency of *F57* and
274 *IS900* sequences during PCR.

275 Magnetic beads combined with phage D29 infection detected viable *M. smegmatis* from urine
276 suspension containing at least 10^3 CFU/mL. Since our experiments have shown that centrifugation can
277 affect the viability of *M. smegmatis* cells from a suspension with 10^6 CFU/mL to the point where no
278 viable cells remained, and considering that the decontamination of samples may damage the
279 mycobacteria and reduce the sensitivity of phage-based assays for detecting mycobacteria cells (61), our
280 method based on magnetic bead-mediated capture followed by phage D29 infection shows potential to
281 detect low amounts of MAP cells from cow's milk, and even MTB from urine samples, within 24 h.
282 However, since the number of plaques (02 PFU) obtained from urine suspension spiked with 2 x 10^3
283 CFU/mL of *M. smegmatis* may raise questions on reliability, the addition of an incubation step, enabling
284 growth of recovered *M. smegmatis* at 37°C for 24 h, prior to phage addition could improve sensitivity by
285 increasing the number of cells infected by phages, and by extension the number of plaques on the
286 indicator plate. Despite that the final concentration of phages in PBS and urine suspensions (2.5 x 10^7
287 PFU/mL) should ensure an infection rate of 1% (maximum infection rate) (44), the presence of beads in
288 PBS and of urea in urine would explain the low infection rate (<1%) of cells by phages. Beads may
289 interfere with the interaction between cells and phages, while the amino group of urea may interact
290 with Tosyl groups and reduce the number of cells captured by beads.

291 Another variable that positively contributed to the capture of mycobacteria by beads was
292 related to the technical steps required to take aliquots of beads in stock suspension, remove the
293 supernatant after capturing beads coupled to mycobacteria and washing beads. Before taking an aliquot
294 of the stock beads suspension, it was homogenized by pipetting 3 – 4 times carefully. The supernatant
295 was removed without taking out the microtube from the magnetic rack to avoid removing beads
296 coupled to mycobacteria. PBS washing was done carefully despite the fact that strong interactions are
297 expected between beads and mycobacteria surface proteins. PBS was added slowly and carefully along
298 the inner wall of the microtube and not directly onto the beads. The microtube should be slightly
299 inverted 5 times to ensure that all beads were distributed homogenously and were not making visible
300 clumps that could carry debris present in the sample. The use of 2-mL microcentrifuge tubes with
301 attached flat top cap ensured a) homogenous mixing of suspensions for increasing interaction between
302 beads and cells, b) removal of supernatant while the tube was placed in the magnetic rack, and c)
303 addition of PBS to the tube for washing cells coupled to beads.
304 A previous study (62) demonstrating the use of Tosyl-activated Dynabeads™ M-280 coupled to
305 Gp10, a recombinant lysin from the mycobacteriophage L5, in combination with qPCR, did not present
306 data about the capture efficiency of uncovered magnetic beads. Another study (47) showed that other
307 types of magnetic beads coupled to peptides and antibodies, and complemented with phages, detected
308 approximately 0.03% and 0.003% of 10^3 to 10^4 CFU/mL MAP cells from milk, respectively. However,
309 unlike the method presented in the present study, which recovered MAP cells directly from milk, the
310 MAP cells were sedimented from milk by centrifugation and were then transferred to one microtube
311 containing 7H9-OADC media for magnetic separation of MAP cells. Moreover, the authors (47) showed
312 that other types of uncoated beads recovered a moderate percentage (around 10%) of nontarget
313 mycobacteria.
314 A limitation of the present study was that MAP cells recovered by beads were not cultured.
315 Although the two log10 lower counts of MAP cells between theoretical concentration determined using
316 OD_{600} and *F57*-qPCR can be explained by clumps present in suspension and not detected using optical
317 density, our results were in line with another study (42) that also reported a difference of two log10
318 counts between colony counts on culture media and *F57*-qPCR. Counting recovered cells before qPCR
319 could further help infer the presence of death and live cells recovered by beads and centrifugation.
320 Another variable that could not be controlled in this study was the effect of calcium as chelating agent in
321 milk. Removing calcium from milk could reduce the formation of MAP clumps and keep a homogenous
322 distribution of cells in suspension to be captured by beads. This study did not evaluate the effect of less

amount of feces (1 g (19,63), 2g (64,65)) on the capture of MAP cells; even though this could reduce the presence and impact of inhibitors. The evaluation of these variables, and the use of target magnetic beads, in further studies would further define the potential to use untargeted magnetic beads to capture MAP cells from milk and feces.

The results of this study encourage further effort to evaluate the utility of Dynabeads® M-280 Tosylactivated, with and without specific ligand, in real animals' samples with a presumptive diagnosis of paratuberculosis. Likewise, the use of these untargeted beads, in combination with a microbiological (e.g., DS6A phage, culture media, colorimetric method) or a molecular test (e.g., HAIN-MTBDRplus, HAIN-MTBDRsl, GeneXpert), would have high potential to be used as a simple and rapid tool for diagnosis of TB.

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Author contributions:

GRP designed the study and conducted the experiments. GRP and DS led the data analysis and interpretation with input from all authors. GRP wrote the manuscript and all authors edited the manuscript.

Declaration of competing interest:

None.

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References

1. Baliga S, Murphy C, Sharon L, Shenoy S, Biranthabail D, Weltman H, et al. Rapid method for detecting and differentiating Mycobacterium tuberculosis complex and non-tuberculous mycobacteria in sputum by fluorescence in situ hybridization with DNA probes. Int J Infect Dis. 2018;75:1–7.
2. Palmer M V., Welsh MD, Hostetter JM. Mycobacterial diseases of animals. Vet Med Int. 2011;2011:292469.

355 3. Li H, Turhan V, Chokhani L, Stratton CW, Dunbar SA, Tang YW. Identification and differentiation
356 of clinically relevant Mycobacterium species directly from acid-fast Bacillus-positive culture
357 broth. J Clin Microbiol. 2009;47:3814–20.
358 4. WHO. Global tuberculosis report 2020. 2020. Available from:
359 https://www.who.int/publications/i/item/9789240013131
360 5. Narvaiz de Kantor, Isabel, Kim, Sang Jae, Frieden, Thomas R, Laszlo, Adalbert, Luelmo F et al.
361 Laboratory services in tuberculosis control culture. Part III: Culture. World Health Organization.
362 1998. Available from:
363 https://apps.who.int/iris/bitstream/handle/10665/65942/WHO_TB_98.258_%28part3%29.pdf?
364 sequence=3&isAllowed=y.
365 6. Nikbakhsh N, Bayani M, Siadati S. The value of bronchoalveolar lavage in the diagnosis of
366 sputum smear-negative pulmonary tuberculosis. Iran J Pathol. 2015;10:35–40.
367 7. Baughman RP, Dohn MN, Loudon RG, Frame PT. Bronchoscopy with bronchoalveolar lavage in
368 tuberculosis and fungal infections. Chest. 1991;99:92–7.
369 8. DiNardo AR, Detjen A, Ustero P, Ngo K, Bacha J, Mandalakas AM. Culture is an imperfect and
370 heterogeneous reference standard in pediatric tuberculosis. Tuberculosis. 2016;101:S105–8.
371 9. Ruiz Jiménez M, Guillén Martín S, Prieto Tato LM, Cacho Calvo JB, Álvarez García A, Soto Sánchez
372 B, et al. Induced sputum versus gastric lavage for the diagnosis of pulmonary tuberculosis in
373 children. BMC Infect Dis. 2013;13:222.
374 10. Gopinath K, Singh S. Urine as an adjunct specimen for the diagnosis of active pulmonary
375 tuberculosis. Int J Infect Dis. 2009;13:374–9.
376 11. Peter J, Green C, Hoelscher M, Mwaba P, Zumla A, Dheda K. Urine for the diagnosis of
377 tuberculosis: Current approaches, clinical applicability, and new developments. Curr Opin Pulm
378 Med. 2010;16:262-70.
379 12. Turbawaty DK, Sugianli AK, Soeroto AY, Setiabudiawan B, Parwati I. Comparison of the
380 performance of urinary Mycobacterium tuberculosis antigens cocktail (ESAT6, CFP10, and
381 MPT64) with culture and microscopy in pulmonary tuberculosis patients. Int J Microbiol.
382 2017;2017:3259329.
383 13. Walters E, Demers AM, Van Der Zalm MM, Whitelaw A, Palmer M, Bosch C, et al. Stool culture
384 for diagnosis of pulmonary tuberculosis in children. J Clin Microbiol. 2017;55:3355–65.

385 14. Oramasionwu GE, Heilig CM, Udomsantisuk N, Kimerling ME, Eng B, Nguyen HD, et al. The utility
386 of stool cultures for diagnosing tuberculosis in people living with the human immunodeficiency
387 virus. Int J Tuberc Lung Dis. 2013;17:1023–8.
388 15. Harris NB, Barletta RG. Mycobacterium avium subsp. paratuberculosis in veterinary medicine.
389 Clin Microbiol Rev. 2001;14:489–512.
390 16. de Juan L, Alvarez J, Romero B, Bezos J, Castellanos E, Aranaz A, et al. Comparison of four
391 different culture media for isolation and growth of type II and type I/III Mycobacterium avium
392 subsp. paratuberculosis strains isolated from cattle and goats. Appl Environ Microbiol.
393 2006;72:5927–32.
394 17. Rangel SJ, Paré J, Doré E, Arango JC, Côté G, Buczinski S, et al. A systematic review of risk factors
395 associated with the introduction of Mycobacterium avium spp. paratuberculosis (MAP) into
396 dairy herds. Can Vet J. 2015;56:169-77.
397 18. Kent P T KGP. Public health mycobacteriology: a guide for the level III laboratory. US Department
398 of Public Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta,
399 GA,. 1985. p. 31-67.
400 19. Sattar A, Zakaria Z, Abu J, Aziz SA, Gabriel RP. Evaluation of six decontamination procedures for
401 isolation of Mycobacterium avium complex from avian feces. PLoS One. 2018;13:e0202034.
402 20. Ganoza CA, Ricaldi JN, Chauca J, Rojas G, Munayco C, Agapito J, et al. Novel hypertonic saline-
403 sodium hydroxide (HS-SH) method for decontamination and concentration of sputum samples
404 for Mycobacterium tuberculosis microscopy and culture. J Med Microbiol. 2008;57:1094–8.
405 21. Chatterjee M, Bhattacharya S, Karak K, Dastidar SG. Effects of different methods of
406 decontamination for successful cultivation of Mycobacterium tuberculosis. Indian J Med Res.
407 2013;138:541–8.
408 22. Addise D, Bitew A, Yaregal Z, Yenew B, Mollalign H, Diriba G, et al. Effect of 1.5% sodium
409 hydroxide final concentration on recovery rate of mycobacterial species and decontamination of
410 other bacterial and fungal contaminants on sputum. Ethiop J public Heal Nutr. 2016;1:57.
411 23. Parsons LM, Somoskövi Á, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory
412 diagnosis of tuberculosis in resource-poor countries: Challenges and opportunities. Clin
413 Microbiol Rev. 2011;24:314-50.
414 24. Hsih H-Y, Tsen H-Y. Combination of immunomagnetic separation and polymerase chain reaction
415 for the simultaneous detection of Listeria monocytogenes and Salmonella spp. in food samples.
416 J Food Prot. 2001;64:1744–50.

417 25. Rijpens N, Herman L, Vereecken F, Jannes G, De Smedt J, De Zutter L. Rapid detection of stressed
418 Salmonella spp. in dairy and egg products using immunomagnetic separation and PCR. Int J Food
419 Microbiol. 1999;46:37–44.
420 26. Karch H, Janetzki-Mittmann C, Aleksic S, Datz M. Isolation of enterohemorrhagic Escherichia coli
421 O157 strains from patients with hemolytic-uremic syndrome by using immunomagnetic
422 separation, DNA-based methods, and direct culture. J Clin Microbiol. 1996;34:516–9.
423 27. Possé B, De Zutter L, Heyndrickx M, Herman L. Quantitative isolation efficiency of O26, O103,
424 O111, O145 and O157 STEC serotypes from artificially contaminated food and cattle faeces
425 samples using a new isolation protocol. J Appl Microbiol. 2008;105:227–35.
426 28. Ghodbane R, Drancourt M. Magnetic bead protocol for culturing mycobacterium tuberculosis
427 from sputum specimens. J Clin Microbiol. 2013;51:1578–9.
428 29. Intorasoot S, Tharinjaroen CS, Phunpae P, Butr-Indr B, Anukool U, Intachai K, et al. Novel
429 potential diagnostic test for Mycobacterium tuberculosis complex using combined
430 immunomagnetic separation (IMS) and PCR-CTPP. J Appl Microbiol. 2016;121:528–38.
431 30. Yoshimatsu S, Kato-Matsumaru T, Aono A, Chikamatsu K, Yamada H, Mitarai S. Factors
432 contribute to efficiency of specimen concentration of Mycobacterium tuberculosis by
433 centrifugation and magnetic beads. Int J Mycobacteriology. 2015;4:245–9.
434 31. Albert H, Ademun PJ, Lukyamuzi G, Nyesiga B, Manabe Y, Joloba M, et al. Feasibility of magnetic
435 bead technology for concentration of mycobacteria in sputum prior to fluorescence microscopy.
436 BMC Infect Dis. 2011;11:1–9.
437 32. Saad J, Loukil A, Drancourt M. Bead-captured Mycobacterium tuberculosis for next-generation
438 sequencing diagnosis of uncultured tuberculosis. Eur J Clin Microbiol Infect Dis. 2020;39:205-7.
439 33. Foddai A, Elliott CT, Grant IR. Maximizing capture efficiency and specificity of magnetic
440 separation for Mycobacterium avium subsp. paratuberculosis cells. Appl Environ Microbiol.
441 2010;76:7550–8.
442 34. Husakova M, Dziedzinska R, Slana I. Magnetic separation methods for the detection of
443 Mycobacterium avium subsp. paratuberculosis in various types of matrices: A review. Biomed
444 Res Int. 2017;2017:5869854.
445 35. Ekong MO. A review on laboratory diagnosis of Mycobacterium tuberculosis. Asian J Res Infect
446 Dis. 2019;2:1–7.

447 36. Singh U, Arutyunov D, Basu U, Santos Seckler H Dos, Szymanski CM, Evoy S. Mycobacteriophage
448 lysin-mediated capture of cells for the PCR detection of Mycobacterium avium subspecies
449 paratuberculosis. Anal Methods. 2014;6:5682–9.
450 37. Invitrogen. Magnetic Separation Technology Surface-activated Dynabeads ® A wide product
451 portfolio for flexible molecular separations [Internet]. [cited 2019 Apr 26]. Available from:
452 https://assets.thermofisher.com/TFS-Assets/LSG/brochures/Surface_Activated_Dynabeads.PDF
453 38. He Z, De Buck J. Localization of proteins in the cell wall of Mycobacterium avium subsp.
454 paratuberculosis K10 by proteomic analysis. Proteome Sci. 2010;8:21.
455 39. Beatty WL, Russell DG. Identification of mycobacterial surface proteins released into subcellular
456 compartments of infected macrophages. Infect Immun. 2000;68:6997–7002.
457 40. He Z, De Buck J. Cell wall proteome analysis of Mycobacterium smegmatis strain MC2 155. BMC
458 Microbiol. 2010;10:121.
459 41. Mortier RAR, Barkema HW, Bystrom JM, Illanes O, Orsel K, Wolf R, et al. Evaluation of age-
460 dependent susceptibility in calves infected with two doses of Mycobacterium avium subspecies
461 paratuberculosis using pathology and tissue culture. Vet Res. 2013;44:1–9.
462 42. Kralik P, Beran V, Pavlik I. Enumeration of Mycobacterium avium subsp. paratuberculosis by
463 quantitative real-time PCR, culture on solid media and optical densitometry. BMC Res Notes.
464 2012;5:114.
465 43. Sevilla IA, Molina E, Elguezabal N, Pérez V, Garrido JM, Juste RA. Detection of mycobacteria,
466 Mycobacterium avium subspecies, and Mycobacterium tuberculosis complex by a novel
467 tetraplex real-time PCR assay. J Clin Microbiol. 2015;53:930–40.
468 44. McNerney R, Kambashi BS, Kinkese J, Tembwe R, Godfrey-Faussett P. Development of a
469 bacteriophage phage replication assay for diagnosis of pulmonary tuberculosis. J Clin Microbiol.
470 2004;42:2115–20.
471 45. Klann AG, Belanger AE, Abanes-de Mello A, Lee JY, Hatfull GF. Characterization of the dnaG locus
472 in Mycobacterium smegmatis reveals linkage of DNA replication and cell division. J Bacteriol.
473 1998;180:65–72.
474 46. Chauca JA, Palomino JC, Guerra H. Evaluation of rifampicin and isoniazid susceptibility testing of
475 Mycobacterium tuberculosis by a mycobacteriophage D29-based assay. J Med Microbiol.
476 2007;56:360–4.
477 47. Foddai A, Elliott CT, Grant IR. Maximizing capture efficiency and specificity of magnetic
478 separation for Mycobacterium avium subsp. paratuberculosis. Cells. 2010;76:7550–8.

479 48. Ratnam S, March SB. Effect of relative centrifugal force and centrifugation time on
480 sedimentation of mycobacteria in clinical specimens. J Clin Microbiol. 1986;23:582–5.
481 49. WAYNE LG. The use of millipore filters in clinical laboratories. Am J Clin Pathol. 1957;28:565–7.
482 50. Smithwick RW, Stratigos CB. Acid-fast microscopy on polycarbonate membrane filter sputum
483 sediments. J Clin Microbiol. 1981;13:1109–13.
484 51. Ratnam S, March SB. Effect of relative centrifugal force and centrifugation time on
485 sedimentation of mycobacteria in clinical specimens. J Clin Microbiol. 1986;23:582–5.
486 52. Kennedy JA, Baron VO, Hammond RJH, Sloan DJ, Gillespie SH. Centrifugation and
487 decontamination procedures selectively impair recovery of important populations in
488 Mycobacterium smegmatis. Tuberculosis. 2018;112:79–82.
489 53. Peterson BW, Sharma PK, van der Mei HC, Busscher HJ. Bacterial cell surface damage due to
490 centrifugal compaction. Appl Environ Microbiol. 2012;78:120–5.
491 54. Górska-Warsewicz H, Rejman K, Laskowski W, Czeczotko M. Milk and dairy products and their
492 nutritional contribution to the average polish diet. Nutrients. 2019;11:1771.
493 55. Black CE, Berg RL, Urquhart AC. 24-Hour urinary calcium in primary hyperparathyroidism. Clin
494 Med Res. 2013;11:219–25.
495 56. Tasara T, Stephan R. Development of an F57 Sequence-based real-time PCR assay for detection
496 of Mycobacterium avium subsp. paratuberculosis in milk. Appl Environ Microbiol.
497 2005;71:5957–68.
498 57. Donaghy JA, Johnston J, Rowe MT. Detection of Mycobacterium avium ssp. paratuberculosis in
499 cheese, milk powder and milk using IS900 and F57-based qPCR assays. J Appl Microbiol.
500 2011;110:479–89.
501 58. Cunha MV., Rosalino LM, Leão C, Bandeira V, Fonseca C, Botelho A, et al. Ecological drivers of
502 Mycobacterium avium subsp. paratuberculosis detection in mongoose (Herpestes ichneumon)
503 using IS900 as proxy. Sci Rep. 2020;10:1–14.
504 59. Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, et al. Complex
505 polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J Clin Microbiol.
506 1997;35:995–8.
507 60. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors - occurrence, properties and
508 removal J Appl Microbiol. 2012;113:1014-26.

509 61. Kalantri S, Pai M, Pascopella L, Riley L, Reingold A. Bacteriophage- based tests for the detection
510 of Mycobacterium tuberculosis in clinical specimens: A systematic review and meta-analysis.
511 BMC Infect Dis. 2005;5:59.
512 62. Singh U, Arutyunov D, Basu U, Dos H, Seckler S, Szymanski CM, et al. Mycobacteriophage lysin-
513 mediated capture of cells for the PCR detection of Mycobacterium avium subspecies
514 paratuberculosis. Anal Meth 2014; 6:5682-9.
515 63. Zhang MZ, Zhang S. An efficient DNA extraction method for polymerase chain reaction-based
516 detection of Mycobacterium avium subspecies paratuberculosis in bovine fecal samples. J Vet
517 Diagn Invest. 2011;23:41–8.
518 64. Keller SM, Stephan R, Kuenzler R, Meylan M, Wittenbrink MM. Comparison of fecal culture and
519 F57 real-time polymerase chain reaction for the detection of Mycobacterium avium subspecies
520 paratuberculosis in Swiss cattle herds with a history of paratuberculosis. Acta Vet Scand.
521 2014;56:68.
522 65. Bauman CA, Jones-Bitton A, Jansen J, Kelton D, Menzies P. Evaluation of fecal culture and fecal
523 RT-PCR to detect Mycobacterium avium ssp. paratuberculosis fecal shedding in dairy goats and
524 dairy sheep using latent class Bayesian modeling. BMC Vet Res. 2016;12:212.

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527

528 **Table 1: *Escherichia coli* and *Mycobacterium smegmatis* remaining in supernatant after centrifugation and magnetic beads**

529 **methods.**

Method	***% E. coli* (mean, ±SD) in supernatant from (CFU/mL)**	***% M. smegmatis* (mean, ±SD) in supernatant from (CFU/mL)**			
	$1,8 \times 10^7$	**1.68×10^7**	**1.68×10^6**	**1.68×10^5**	**1.68×10^4**
Centrifugation	0	0.66 (1.12×10^5, $\pm 7.64 \times 10^3$)	0	0	0
Magnetic beads	94.63 (1.7×10^7, $\pm 5.75 \times 10^4$)	4.04 (6.79×10^5, $\pm 2.16 \times 10^4$)	3.07 (5.17×10^4, $\pm 2.96 \times 10^3$)	6.71 (1.13×10^4, $\pm 9.57 \times 10^2$)	3.69 (6.21×10^2, $\pm 1.22 \times 10^2$)

530

531 **Table 2: Recovery and detection of MAP cells by magnetic beads and *F57*-qPCR**

PBS suspension	OD_{600}, 1.0 (theoretical)[a] CFU/mL (±SD)	Initial MAP cells concentration CFU/mL[b]	MAP cells recovered by centrifugation* and quantified by *F57*-qPCR		MAP cells recovered by beads and quantified by *F57*-qPCR	
			CFU/mL (±SD)	Ct	CFU/mL (±SD)	Ct
First	$3.17x10^7$ ($\pm 2.02x10^7$)	$2.93x10^9$	$2.8x10^9$ ($\pm 4.1x10^8$)	17.89[c]	$3.2x10^9$ ($\pm 4.5x10^8$)	17.68[c]
Second	$3.17x10^5$ ($\pm 2.02x10^5$)	$1.43x10^7$	$1.4x10^7$ ($\pm 1.8x10^6$)	25.43[d]	$3.9x10^7$ ($\pm 5.5x10^6$)	23.94[d]

532 [a] Approximation of CFU in each suspension was assessed by OD_{600} measurements.

533 [b] Initial concentration (100%) of MAP cells in the first and second PBS suspensions was estimated by detecting *F57* sequence from MAP cells

534 recovered by centrifugation (in theory 95%)*.

535 [c] *F57*-qPCR showed that there was a statistically significant difference ($p=0.04$) in the recovery of MAP cells by magnetic beads and

536 centrifugation.

537 [d] *F57*-qPCR showed that there was a statistically significant difference ($p<0.0001$) in the recovery of MAP cells by magnetic beads and

538 centrifugation.

539 **Table 3:** Recovery rate of *M. avium paratuberculosis* from milk, urine, and feces sample

Medium	Initial Concentration (CFU/mL)	*F57*-qPCR			*IS900*-qPCR
		CFU/mL (mean; ± SD)	Recovery (%)	Ct (mean; ±SD)	Ct (mean; ±SD)
Milk	$8.92x10^9$	$5.45x10^8$; ±$8.31x10^7$	6.11	20.45; ±0.22	16.30; ±0.18
	$6.25x10^7$	$3.73x10^6$; ±$1.01x10^6$	5.96	27.61; ±0.38	23.89; ±0.13
	$6.11x10^6$	$3.80x10^4$; ±$2.44x10^4$	0.62	36.44; ±1.11	32.14; ±1.74
	$4.92x10^5$	UND	NA	NA	35.45; ±0.78
	$5.19x10^4$	UND	NA	NA	36.59; NA
Urine	$8.92x10^9$	$2.76x10^9$; ±$4.46x10^8$	30.93	18.12; ±0.23	13.84; ±0.08
	$6.25x10^7$	$2.70x10^7$; ±$6.85x10^6$	43.25	24.77; ±0.34	20.53; ±0.06
	$6.11x10^6$	$2.72x10^6$; ±$2.81x10^6$	44.56	31.72; ±2.71	26.63; ±2.93
	$4.92x10^5$	$4.23x10^4$; ±$2.70x10^4$	8.60	36.24; ±0.82	32.42; ±2.01
	$5.19x10^4$	UND	NA	NA	35.74; ±1.18
Feces	$8.92x10^9$	$5.93x10^7$; ±$1.54x10^7$	0.66	28.27; ±0.35	ND
	$6.25x10^7$	UND	UND	UND	ND
	$6.11x10^6$	UND	UND	UND	ND
	$4.92x10^5$	UND	UND	UND	ND
	$5.19x10^4$	UND	UND	UND	ND

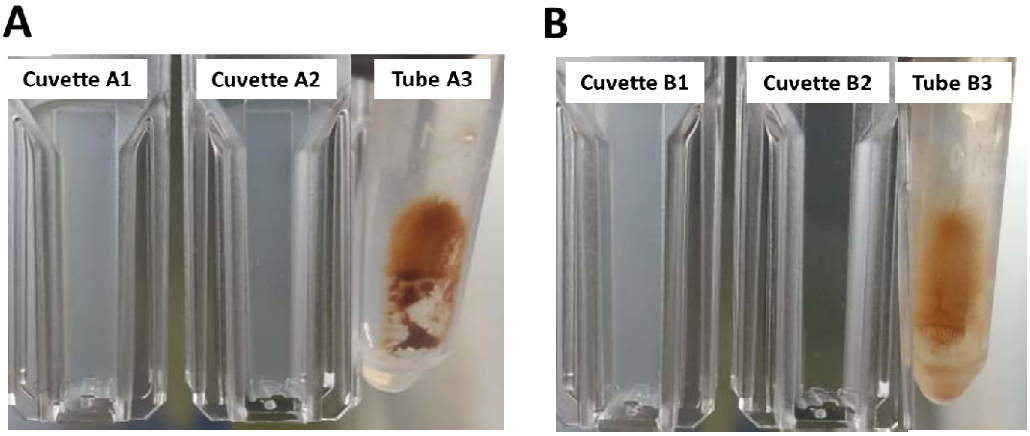
540

541 **Table 4: Detection of *M. smegmatis* from PBS and urine by beads and phage D29**

	PFU (mean* ±SD) detected from spiked *M. smegmatis* (CFU/mL)	
	2×10^5 CFU/mL	2×10^3 CFU/mL
PBS (control)	259.33 ±34.08	12.33 ±2.52
Urine	56 ±8.54	2 ±1

542 *Mean of three experiments.

543



544

545 **FIG 1 Capture of *E. coli* and *M. smegmatis* cells in PBS suspension by magnetic beads.**

546 A. Capturing *E. coli* in PBS by beads. Cuvette A1: Concentration (1,8 x 10^7 CFU/ml) of *E. coli* before
547 capture; cuvette A2: Concentration (1,7 x 10^7 CFU/ml) of *E. coli* suspension after beads capture.
548 Microtube A3: Magnetic beads bound to *E. coli* cells and separated in a magnetic rack; B. Capturing *M.*
549 *smegmatis* in PBS by beads. Cuvette B1: Concentration (1,68 x 10^7 CFU/ml) of *M. smegmatis* suspension
550 before beads capture; cuvette B2: Concentration (6.56 x 10^5 CFU/ml) of *M. smegmatis* suspension after
551 beads capture; microtube B3: Magnetic beads bound to *M. smegmatis* cells and separated in a magnetic
552 rack.

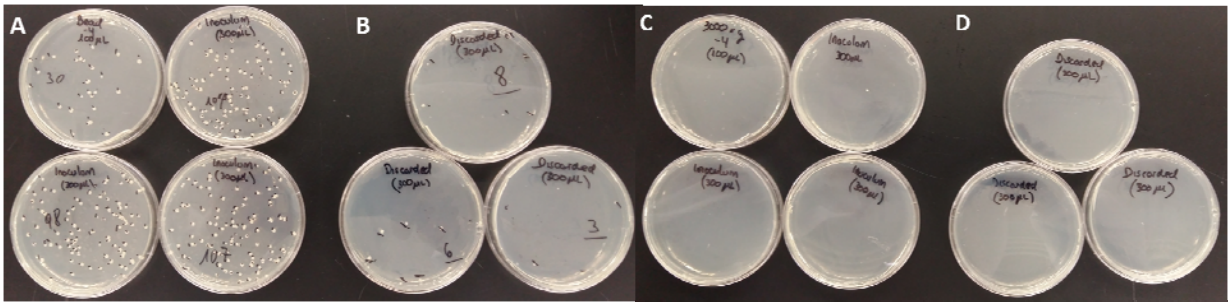


FIG 2 *M. smegmatis* cells recovered by magnetic beads and centrifugation. From a suspension of 1.68×10^4 CFU/mL of *M. smegmatis*, A and B show cells captured (precipitated) and not captured (supernatant) by beads, respectively. As per the *M. smegmatis* cells remaining in the supernatant (3.7%), the estimated number of cells recovered from the beads was 96.3%. C and D show that no viable cells were recovered from the supernatant or pellet after centrifugation.

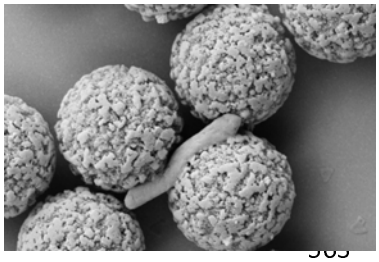
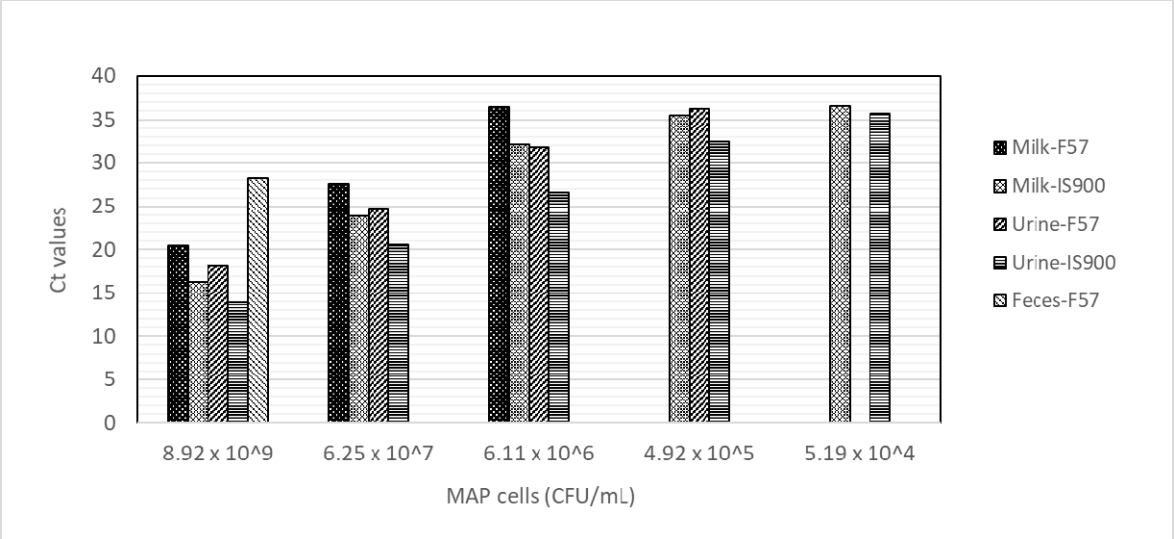


FIG 3 A scanning electron microscope image of a M. smegmatis cell captured by magnetic beads.

Capturing *M. smegmatis* cell in PBS by magnetic beads.

567



568

569 **FIG 4 Detection of the *F57* sequence by qPCR from MAP cells recovered by magnetic beads.**

570 Ct values showed that magnetic beads captured MAP cells from milk, urine, and feces. From feces, milk,

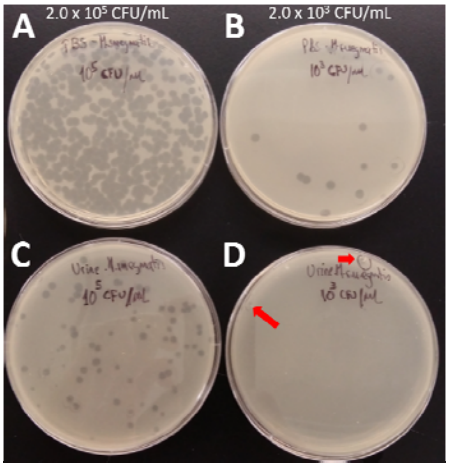
571 and urine, *F57* sequences were detected up to 8.92 x 10^9, 6.11 x 10^6, and 4.92 x 10^5 CFU/mL,

572 respectively. *IS900*, unlike *F57*, showed better sensitivity to detect lowest (10^4 CFU/mL) MAP cells

573 concentration from milk and urine. Other feces suspensions containing lower concentrations were not

574 detected by qPCR. Detection of IS sequence from feces sample was not done.

575

576 

577 **FIG 5 Detection of *M. smegmatis* from urine suspensions by mycobacteriophage D29.**

578 Detection of *M. smegmatis* captured from PBS suspensions (A and B) and urine suspensions (C and D) by

579 mycobacteriophage D29 in 24 hours.