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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17 **ABSTRACT**

18 Untargeted magnetic beads (Dynabeads® M-280 Tosylactivated) were evaluated to capture
19 *Mycobacterium smegmatis* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from spiked
20 feces, milk, and urine. Untargeted magnetic beads added to the spiked samples were slightly mixed for 1
21 hour and separated in a magnetic rack for further detection. Beads recovered more *M. smegmatis* cells
22 from PBS suspension than the typical centrifugation method; these results were confirmed by the
23 recovery of 96.31% of 1.68 x 10^4 CFU/mL viable *M. smegmatis* by beads and 0% by centrifugation.
24 Likewise, the *F57*-qPCR detection of MAP cells was different whether they were recovered by beads or
25 centrifugation; cycle threshold (Ct) was lower ($p<0.05$) for the detection of MAP cells recovered by
26 beads than centrifugation, indicative of higher sensitivity. Magnetic separation of MAP cells from milk,
27 urine, and feces specimens were detected by amplifying *F57* and *IS900* sequences. Milk proteins were
28 denatured by Proteinase k before capturing MAP cells by magnetic beads. Ct values demonstrated that
29 beads captured no less than 10^9 CFU/mL from feces and no less than 10^4 CFU/mL from milk and urine
30 suspensions. In another detection strategy, *M. smegmatis* coupled to magnetic beads were infected by
31 mycobacteriophage D29. Plaque forming units were observed after 24 h of incubation from urine
32 samples containing 2 x 10^5 and 2 x 10^3 CFU/mL *M. smegmatis*. The results of this study provide a
33 promising basis for the use of untargeted beads as a simple tool for diagnosis of Johne's disease and
34 other mycobacterial diseases such as tuberculosis.

35 **INTRODUCTION**

36

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

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

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

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

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

99 **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 **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 **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 **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 **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 **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: (ng DNA × 6,023 × 10^{23})/(4,829,781 × 10^9 × 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 **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 x 10^5 and
180 2.0 x 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 **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 **RESULTS**

196

197 ***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 **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 ***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 **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 **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 2×10^3
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 (2.5×10^7
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
323 amount of feces (1 g (19,63), 2g (64,65)) on the capture of MAP cells; even though this could reduce the
324 presence and impact of inhibitors. The evaluation of these variables, and the use of target magnetic
325 beads, in further studies would further define the potential to use untargeted magnetic beads to
326 capture MAP cells from milk and feces.
327 The results of this study encourage further effort to evaluate the utility of Dynabeads® M-280
328 Tosylactivated, with and without specific ligand, in real animals' samples with a presumptive diagnosis
329 of paratuberculosis. Likewise, the use of these untargeted beads, in combination with a microbiological
330 (e.g., DS6A phage, culture media, colorimetric method) or a molecular test (e.g., HAIN-MTBDRplus,
331 HAIN-MTBDRsl, GeneXpert), would have high potential to be used as a simple and rapid tool for
332 diagnosis of TB.
333
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337
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517

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

519 **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, $\pm 1.22 \times 10^2$)

520

521 **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	$3.17x10^7$ (±$2.02x10^7$)	$2.93x10^9$	$2.8x10^9$ (±$4.1x10^8$)	17.89[c]	$3.2x10^9$ (±$4.5x10^8$)	17.68[c]
Second	$3.17x10^5$ (±$2.02x10^5$)	$1.43x10^7$	$1.4x10^7$ (±$1.8x10^6$)	25.43[d]	$3.9x10^7$ (±$5.5x10^6$)	23.94[d]

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

523 [b] Initial concentration (100%) of MAP cells in the first and second PBS suspensions was estimated by detecting *F57* sequence from MAP cells
524 recovered by centrifugation (in theory 95%)*.

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

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

529 **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	$8.92x10^9$	$5.45x10^8$; $\pm 8.31x10^7$	6.11	20.45; ±0.22	16.30; ±0.18
	$6.25x10^7$	$3.73x10^6$; $\pm 1.01x10^6$	5.96	27.61; ±0.38	23.89; ±0.13
	$6.11x10^6$	$3.80x10^4$; $\pm 2.44x10^4$	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	$8.92x10^9$	$2.76x10^9$; $\pm 4.46x10^8$	30.93	18.12; ±0.23	13.84; ±0.08
	$6.25x10^7$	$2.70x10^7$; $\pm 6.85x10^6$	43.25	24.77; ±0.34	20.53; ±0.06
	$6.11x10^6$	$2.72x10^6$; $\pm 2.81x10^6$	44.56	31.72; ±2.71	26.63; ±2.93
	$4.92x10^5$	$4.23x10^4$; $\pm 2.70x10^4$	8.60	36.24; ±0.82	32.42; ±2.01
	$5.19x10^4$	UND	NA	NA	35.74; ±1.18
Feces	$8.92x10^9$	$5.93x10^7$; $\pm 1.54x10^7$	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

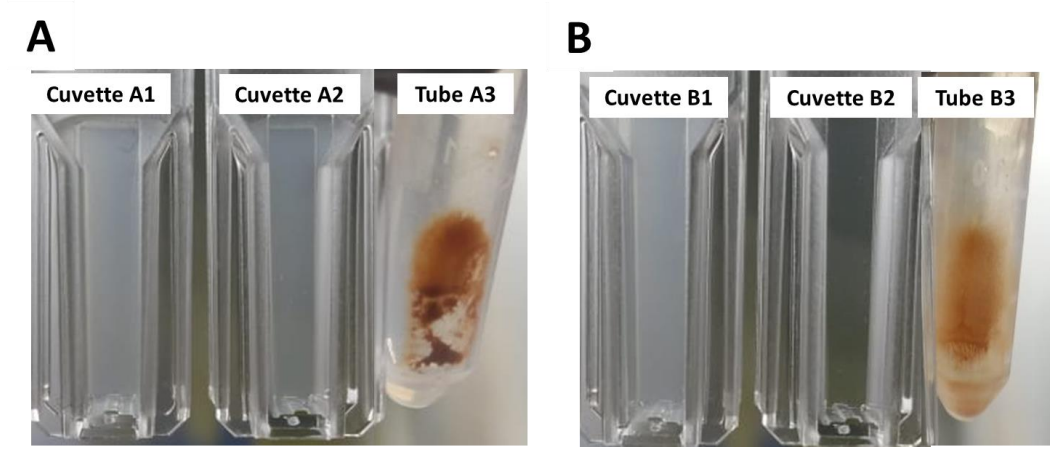
530

531 **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

532 *Mean of three experiments.

533



534

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

536 A. Capturing *E. coli* in PBS by beads. Cuvette A1: Concentration (1,8 x 10^7 CFU/ml) of *E. coli* before
537 capture; cuvette A2: Concentration (1,7 x 10^7 CFU/ml) of *E. coli* suspension after beads capture.
538 Microtube A3: Magnetic beads bound to *E. coli* cells and separated in a magnetic rack; B. Capturing *M.*
539 *smegmatis* in PBS by beads. Cuvette B1: Concentration (1,68 x 10^7 CFU/ml) of *M. smegmatis* suspension
540 before beads capture; cuvette B2: Concentration (6.56 x 10^5 CFU/ml) of *M. smegmatis* suspension after
541 beads capture; microtube B3: Magnetic beads bound to *M. smegmatis* cells and separated in a magnetic
542 rack.

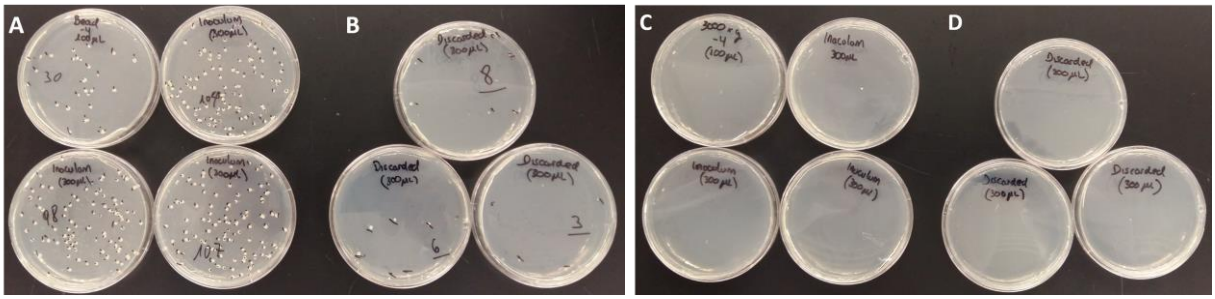
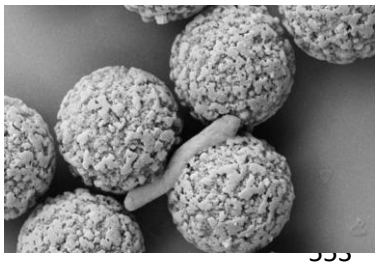


FIG 2 *M. smegmatis* cells recovered by magnetic beads and centrifugation. From a suspension of 1.68×10^4 CFU/mL of *M. smegmatis*, A and B show cells captured (precipitated) and not captured (supernatant) by beads, respectively. As per the *M. smegmatis* cells remaining in the supernatant (3.7%), the 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.

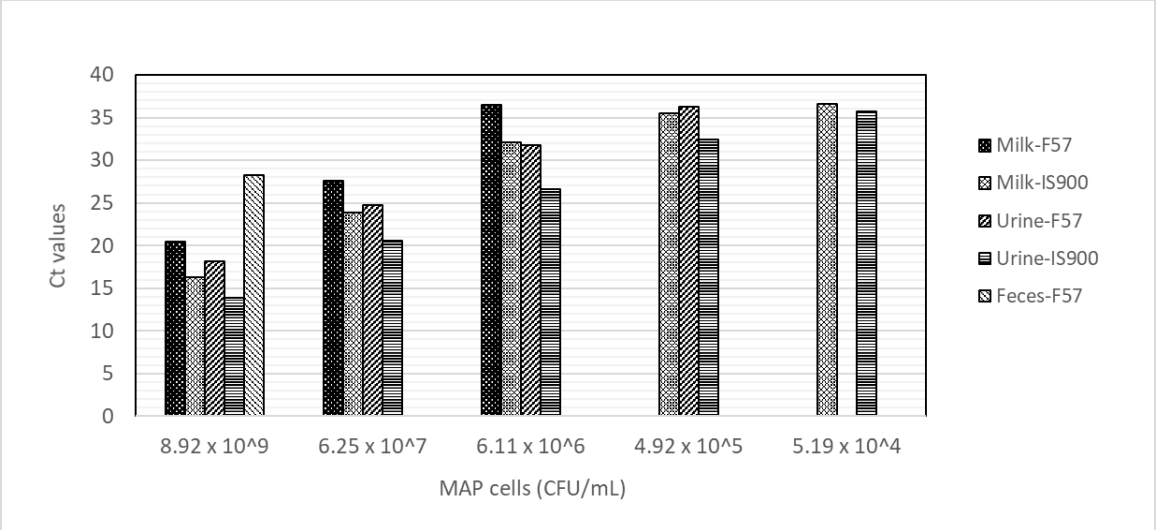


553

554 **FIG 3 Electron microscopy of *M. smegmatis* cell captured by magnetic beads.** Capturing *M. smegmatis*

555 cell in PBS by magnetic beads.

556



557

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

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

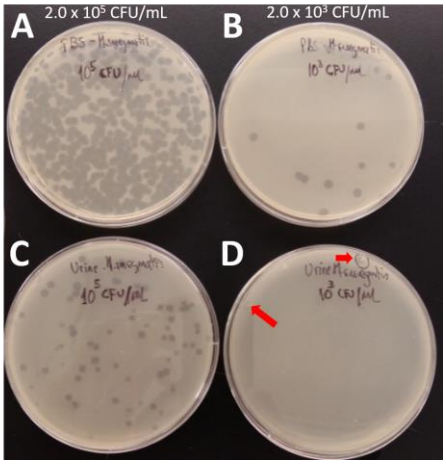
560 and urine, *F57* sequences were detected up to 8.92 x 10^9, 6.11 x 10^6, and 4.92 x 10^5 CFU/mL,

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

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

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

564



565

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

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

568 mycobacteriophage D29 in 24 hours.