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

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

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

66 Tosyl-activated magnetic beads (e.g., Dynabeads M-280 Tosylactivated) can bind covalently to
67 primary amine and sulfhydryl groups in proteins and peptides (37). Importantly, some surface proteins
68 have been identified on mycobacteria (38–40). As per their characteristics, we postulate that Tosyl-

69 activated magnetic beads may interact with these surface proteins enabling the capture of *M.*
70 *smegmatis* and MAP cells in suspension. Because the use of untargeted beads to capture MAP cells
71 would diminish laboratory work and costs, and would increase affordability in countries with a high
72 burden of TB and JD, this study determined the potential of Tosyl-activated Dynabeads M-280
73 (untargeted magnetic beads) to capture *M. smegmatis* and MAP from spiked urine, feces, and milk
74 samples prior to detection by real-time PCR (qPCR) and phage D29.
75

76 **2. Material and Methods**

77 **2.1. 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 **2.2. Magnetic beads assay**

87 Tosyl-activated Dynabeads™ M-280 (Invitrogen)(37) were used for cell capture. Briefly, 21 µl of
88 beads was transferred to one sterile microcentrifuge tube of 2-ml capacity (microtube) and washed
89 twice with 1 ml of PBS (pH 7.4) for 1 min each time. The microtube was placed on a magnet (Magna grip
90 rack, Millipore) for removing supernatant. Washed beads were then added to microtubes containing 1
91 mL of PBS, milk, urine, and feces suspensions spiked with various levels of *M. smegmatis* or MAP cells.
92 These tubes were placed in a microtube rotator (Thermo Scientific™ Labquake™, Edmonton, AB,
93 Canada) at room temperature for 1 h. The microtubes were then placed on a magnetic rack for 1 min to
94 carefully remove the supernatant to another microtube. Magnetic beads coupled to *M. smegmatis* or
95 MAP were washed twice with 1 mL PBS for 1 min before moving on to the detection assays using qPCR
96 or phage D29.

97 **2.3. Recovery of viable M. smegmatis and E. coli cells by magnetic beads**

98 ***M. smegmatis.*** Suspensions of *M. smegmatis* cells were prepared from a stock. It was adjusted to
99 OD_{600} ~1 (microtube#1), and then serial 10-fold dilutions in PBS were performed (microtube#2,
100 microtube#3, and microtube#4) to obtain different cell concentrations for assays. Determination of

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

118 **2.4. MAP cells DNA extraction**

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

126 **2.5. Quantitative qPCR assay**

127 This procedure was done as per Singh et al. (36). Briefly, qPCR amplification targeting *IS900* and
128 *F57* genes was done using Taqman specific probes and StepOnePlus Real Time PCR System (Applied
129 Biosystems). The 10-µL PCR mixture comprised 5 µL of TaqMan® Universal Master Mix II (Applied
130 Biosystems) with uracil-N-glycosylase (UNG), 200 nM of each primer and 250 nM of fluorogenic probe
131 specific to *IS900* and *F57*; the sequence of the primers and probes used were described elsewhere (36).
132 For each template DNA sample, 4 µL was added in the reaction mixture. qPCR conditions were as

133 follows: 50°C for 2 min for UNG treatment, initial denaturation at 95°C for 10 min, 40 cycles at 95°C for
134 15 sec, and at 60°C for 1 min. Each qPCR amplification targeting *IS900* and *F57* genes was run in
135 triplicate with negative and positive controls. Obtained data were analyzed in the form of threshold
136 cycle (Ct) values.

137 **2.6. Detection of MAP cells by qPCR**

138 This experiment was done to evaluate the recovery of MAP cells by beads and centrifugation
139 through the detection of DNA of the total, viable and non-viable, cells sedimented. Efficiency of beads
140 and centrifugation to capture and sediment cells, respectively, was determined by using two referential
141 MAP concentrations (5 x 10^7 CFU/ml and 5 x 10^5 CFU/mL). Briefly, two serial 10-fold dilutions were
142 prepared from the stock (5 x 10^7 CFU/mL) of MAP suspension in PBS. Two aliquots (1 mL) of the stock
143 were transferred to two microtubes to evaluate the recovery of MAP by centrifugation (control) and
144 magnetic beads. MAP cells recovered by beads and centrifugation (3,000 *x g*, 4°C, 16 min) were analyzed
145 by *F57* qPCR. The same procedure was performed for the second serial dilution containing 5 x 10^5
146 CFU/mL. Each condition was repeated three times.

147 **2.7. Capture of MAP in milk, urine and feces by beads**

148 **From milk and urine suspension.** Three sterile microtubes containing 0.9 mL of PBS (pH 7.4), 0.9 mL of
149 urine from an apparently healthy human or 0.9 ml of skim milk (Dairyland®) were spiked with 0.1 mL of
150 10^8 CFU/mL MAP cells. Proteinase K (Pk) was added to the microtube containing milk and incubated at
151 70°C for 10 min before performing the magnetic beads assay. MAP cells recovered using beads were
152 analyzed by qPCR. The same procedure was applied to other microtubes containing fewer MAP cells
153 (10^6, 10^5, 10^4 CFU/mL). The experiment was run in triplicate and repeated twice.

154 **From feces suspension.** Cow feces of a known MAP-shedding calf (41) was provided by the University of
155 Calgary. Feces suspension was prepared by mixing 3 g of apparently healthy cattle feces and 6 ml of PBS
156 in a 15-ml centrifuge microtube. After keeping the microtube in an upright position for 20 min, 0.9 mL of
157 supernatant was transferred to another microtube and spiked with 0.1 mL of 10^8 CFU/mL MAP
158 suspended in PBS. One microtube with 0.9 mL PBS was spiked with 0.1 mL of 10^8 CFU/mL MAP. Both
159 microtubes were used for magnetic beads assays and analyzed by *F57* qPCR. The experiment was run in
160 triplicate and repeated twice.

161 **2.8. Quantification of MAP cells recovered by beads**

162 MAP cell counts were assessed using qPCR based upon the single copy fragment *F57* (42).
163 Briefly, DNA calibration curves were prepared for each experiment by preparing serial 10-fold dilutions
164 of DNA sample obtained from MAP cell (adjusted to OD_{600} ~1). The initial DNA concentration of the first

165 serial dilution was measured through optical absorbance at 260 nm using a NanoDrop ND 1000
166 spectrophotometer (NanoDrop Technologies Inc.). DNA molecule copy number (reported as equivalent
167 CFU/mL) was determined using the formula: (ng DNA × 6,023 × 10^{23})/(4,829,781 × 10^{9} × 660)(43). The
168 number of *F57* (equivalent CFU) detected in each MAP sediment recovered by beads and control was
169 determined by using the respective threshold cycle (Ct) values.
170 (36)

171 **2.9. Magnetic separation of M. smegmatis from urine for mycobacteriophage D29 infection**

172 The experimental procedure was adjusted as per infection of MTB by phage D29 (44) and
173 doubling time (3 to 4 hours) of *M. smegmatis* (45). Two urine aliquots of 1 ml containing 2.0 x 10^{5} and
174 2.0 x 10^{3} CFU/mL *M. smegmatis* were transferred to two sterile microtubes. *M. smegmatis* captured by
175 beads from urine were detected by phage D29 (44,46) with some modifications. Briefly, *M. smegmatis*
176 cells captured by beads were resuspended in 150 µL of LB with 1 mM $CaCl_2$ (LB-S) and infected with 50
177 µL of phage D29 stock at 10^{8} PFU/mL and 37°C. Extracellular phage D29 was eliminated with 0.03 M
178 ferrous ammonium sulfate (FAS) for 10 min; then 0.5 mL of LB-$CaCl_2$ was added to the microtube to stop
179 FAS virucide activity. The total volume suspension was mixed with 4 mL of sterile molten agar (0.8%) and
180 1 mL of 10^{8} CFU/mL *M. smegmatis* suspension before pouring onto Petri dishes with LB agar. Petri
181 dishes were incubated for 24 h at 37°C and the number of infection plaques was counted. The
182 experiment was repeated 3 times.

183 **2.10. Statistical analyses**

184 Number of *IS900* and *F57* gene copies, determined in triplicate per each sediment recovered by
185 u-TDbeads and control, were plotted and analyzed by the Kruskal-Wallis test (GraphPad Instat, version
186 8; GraphPad Prism, La Jolla, CA). *P*-values < 0.05 were considered statistically significant.
187

188 ***3.* Results**

189 **3.1. Capture of M. smegmatis and E. coli by magnetic beads**

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

197 The effect of the centrifugation and magnetic beads methods on the viability of *M. smegmatis*
198 was confirmed by assessing suspensions at 1.68 x 10^4 CFU/mL (Fig. 2). As per one experiment results, 17
199 CFU of *M. smegmatis* isolated from the supernatant and 337 CFU recovered from sedimented beads,
200 337 CFU compressed 1326 CFU of *M. smegmatis* as consequence of the formation of clumps during the
201 magnetic separation (Fig. 2). Thus, the magnetic beads recovered more viable *M. smegmatis* from the
202 suspension (96%) than the centrifugation (0%). An electron microscopy image of the capture of *M.*
203 *smegmatis* cells by beads is presented in Fig. 3.

204 **3.2. Detection of MAP cells by qPCR**

205 The initial concentration of MAP cells in the first and second PBS suspensions (2.93 x 10^9 and
206 1.43 x 10^7 equivalent CFU/mL, respectively) was estimated by detecting *F57* sequence from all MAP cells
207 collected by centrifugation, which is used to concentrate 95% mycobacteria (18). Using *F57*-qPCR, there
208 was a difference (p=0.04) in recovery of MAP cells between the magnetic beads and centrifugation
209 methods: from the first suspension 2.8 x 10^9 CFU/mL (Ct = 17.89) were recovered by magnetic beads
210 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
211 recovered by magnetic beads and 1.4 x 10^7 CFU/mL (Ct = 25.43) by centrifugation ((p<0.0001); Table 2).

212 **3.3. Milk, urine, and feces suspension.**

213 Ct values demonstrated that magnetic beads captured no less than 10^9 CFU/mL from feces and
214 no less than 10^4 CFU/mL of MAP cells from milk and urine suspensions (Fig. 4). From milk suspensions,
215 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
216 CFU/mL, and 0.62% of 3.80 x 10^4 CFU/mL. Pk was incorporated in milk suspensions as preliminary
217 experiments showed that its activity increased the capture of MAP cells by beads (data not shown).
218 Recovery rates of MAP cells from urine suspensions using *F57*-qPCR was 30.93% of 8.92 x 10^9, 43.25% of
219 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*
220 method, was more sensitive to detect the lowest (10^4 CFU/mL) MAP cells concentration from milk and
221 urine (Fig. 4 and Table 3). From feces, 0.66% of 8.92 x 10^9 CFU/mL MAP cells were captured and
222 detected by beads and *F57*-qPCR (*IS900* was not done). MAP cells in feces suspensions at lower
223 concentrations were not detected.

224 **3.4. Magnetic separation of M. smegmatis from urine for mycobacteriophage D29 infection**

225 The combination of magnetic beads and phage D29 enabled the detection of *M. smegmatis*
226 spiked in urine within 24 h (Fig. 5). Although the number of plaques observed for samples from urine
227 spiked with 2 x 10^5 CFU/mL and 2 x 10^3 CFU/mL *M. smegmatis* was lower than the respective cell counts

228 (Table 4), the PFU detected from the samples at higher cell concentration clearly prove the detection of
229 *M. smegmatis* within 24 h.
230
231 **4. Discussion**
232 Untargeted Tosyl-activated Dynabeads™ M-280 recovered more viable *M. smegmatis* cells
233 (>90%) from spiked PBS suspension with 10^7, 10^6, 10^5 and 10^4 CFU/mL than the typical centrifuge-based
234 method. Capture efficiency was estimated by counting unbound *M. smegmatis* cells (in supernatant)
235 that were not captured nor sedimented by beads. Counting cells sedimented by beads, even after
236 resuspending them in 1 mL before plating (47), could underestimate the real number of cells recovered
237 by beads due to formation of cell clumps during the sedimentation process. With regards to cell
238 viability, untargeted magnetic beads did not affect the viability of *M. smegmatis,* supported by growth
239 on LB agar plate and infection by phage D29. On the other hand, centrifugation had a negative effect on
240 the viability of *M. smegmatis* cells, regardless of cell concentration. Moreover, qPCR detection of MAP
241 cells, after sedimentation by centrifugation and magnetic beads, indicates that the use of the latter
242 would be preferable to precipitate cells from PBS suspension. Although the centrifugation method
243 (3,000 *x g* at 4°C for 16 min) has been established as a referential method for the recovery of 95% of
244 cells from suspension (18,48), we agree with others (49–51) that centrifugation may not be an
245 appropriate method to concentrate specimens containing small numbers of microorganisms. The
246 inefficiency of the centrifuge method may be related to the physiology of the bacteria; low recovery of
247 MAP cells from a stationary culture is expected as the majority of cells have a lower buoyant density due
248 to the presence of lipid bodies (52). Likewise, as others have reported for other bacteria undergoing
249 centrifugation (53), the low number of *M. smegmatis* colonies growing on LB agar may be explained by
250 the damage of some bacterial cells, including DNA, during the centrifugation.
251 Since detection of MAP cells from milk improved after treating samples with Pk, we postulate
252 that Tosyl groups can be blocked by milk proteins which would, therefore, affect the capture of MAP
253 cells. The positive effect of Pk on capture of MAP from milk was not observed in urine nor feces,
254 perhaps, due to the higher protein concentration (1.87%) of milk (54) compared to urine (55) and feces.
255 With regards to the magnetic separation of captured MAP cells from urine and feces, we postulate that
256 the differences observed in qPCR sensitivity when detecting MAP cells in milk, urine and feces can be
257 explained by the number of copies of the targeted genes *F57* (1 per genome) (56,57) and *IS900* (14-20
258 per genome)(57,58). Urea amine group, and other inhibitors such as polysaccharides originated from
259 vegetable material in the diet (59), gut flora, bile salts, and glucolipids (60), may react with Tosyl groups

260 and could explain decreases in the capture of cells by beads. Likewise, these inhibitors could be
261 transferred with the beads coupled to MAP cells and reduce the amplification efficiency of *F57* and
262 *IS900* sequences during PCR.

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

279 Another variable that positively contributed to the capture of mycobacteria by beads was
280 related to the technical steps required to take aliquots of beads in stock suspension, remove the
281 supernatant after capturing beads coupled to mycobacteria and washing beads. Before taking an aliquot
282 of the stock beads suspension, it was homogenized by pipetting 3 – 4 times carefully. The supernatant
283 was removed without taking out the microtube from the magnetic rack to avoid removing beads
284 coupled to mycobacteria. PBS washing was done carefully despite the fact that strong interactions are
285 expected between beads and mycobacteria surface proteins. PBS was added slowly and carefully along
286 the inner wall of the microtube and not directly onto the beads. The microtube should be slightly
287 inverted 5 times to ensure that all beads were distributed homogenously and were not making visible
288 clumps that could carry debris present in the sample. The use of 2-mL microcentrifuge tubes with
289 attached flat top cap ensured a) homogenous mixing of suspensions for increasing interaction between
290 beads and cells, b) removal of supernatant while the tube was placed in the magnetic rack, and c)
291 addition of PBS to the tube for washing cells coupled to beads.

292 A previous study (62) demonstrating the use of Tosyl-activated Dynabeads™ M-280 coupled to
293 Gp10, a recombinant lysin from the mycobacteriophage L5, in combination with qPCR, did not present
294 data about the capture efficiency of uncovered magnetic beads. Another study (47) showed that other
295 types of magnetic beads coupled to peptides and antibodies, and complemented with phages, detected
296 approximately 0.03% and 0.003% of 10^3 to 10^4 CFU/mL MAP cells from milk, respectively. However,
297 unlike the method presented in the present study, which recovered MAP cells directly from milk, the
298 MAP cells were sedimented from milk by centrifugation and were then transferred to one microtube
299 containing 7H9-OADC media for magnetic separation of MAP cells. Moreover, the authors (47) showed
300 that other types of uncoated beads recovered a moderate percentage (around 10%) of nontarget
301 mycobacteria.
302 A limitation of the present study was that MAP cells recovered by beads were not cultured.
303 Although the two log10 lower counts of MAP cells between theoretical concentration determined using
304 OD_{600} and *F57*-qPCR can be explained by clumps present in suspension and not detected using optical
305 density, our results were in line with another study (42) that also reported a difference of two log10
306 counts between colony counts on culture media and *F57*-qPCR. Counting recovered cells before qPCR
307 could further help infer the presence of death and live cells recovered by beads and centrifugation.
308 Another variable that could not be controlled in this study was the effect of calcium as chelating agent in
309 milk. Removing calcium from milk could reduce the formation of MAP clumps and keep a homogenous
310 distribution of cells in suspension to be captured by beads. This study did not evaluate the effect of less
311 amount of feces (1 g (19,63), 2g (64,65)) on the capture of MAP cells; even though this could reduce the
312 presence and impact of inhibitors. The evaluation of these variables, and the use of target magnetic
313 beads, in further studies would further define the potential to use untargeted magnetic beads to
314 capture MAP cells from milk and feces.
315 The results of this study encourage further effort to evaluate the utility of Dynabeads® M-280
316 Tosylactivated, with and without specific ligand, in real animals' samples with a presumptive diagnosis
317 of paratuberculosis. Likewise, the use of these untargeted beads, in combination with a microbiological
318 (e.g., DS6A phage, culture media, colorimetric method) or a molecular test (e.g., HAIN-MTBDRplus,
319 HAIN-MTBDRsl, GeneXpert), would have high potential to be used as a simple and rapid tool for
320 diagnosis of TB.
321
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Author contributions:

GRP designed the study and conducted the experiments. GRP, DS and RZ 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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505 **Table 1. *Escherichia coli* and *Mycobacterium smegmatis* remaining in supernatant after centrifugation and magnetic beads**

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

507

508 **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]

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

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

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

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

516 **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

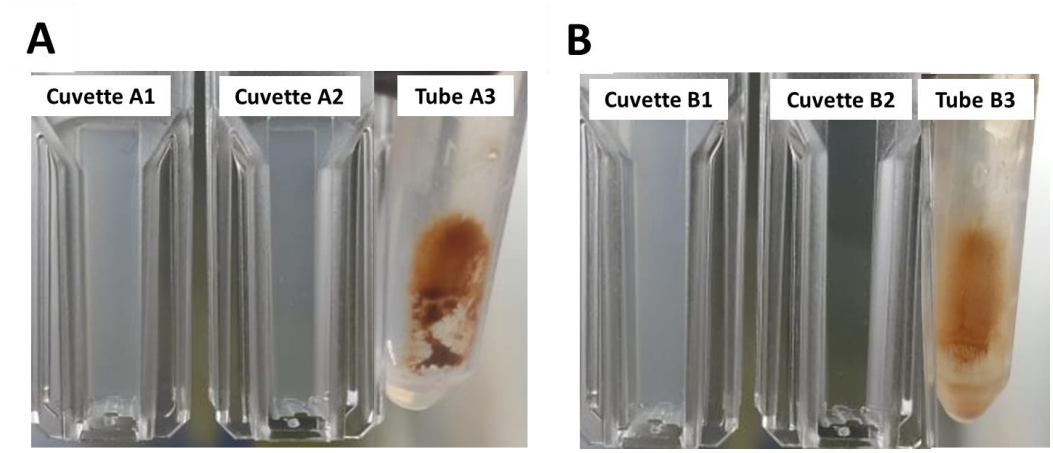
517

518 **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

519 *Mean of three experiments.

520



521

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

523 A. Capturing *E. coli* in PBS by beads. Cuvette A1: Concentration (1,8 x 10^7 CFU/ml) of *E. coli* before
524 capture; cuvette A2: Concentration (1,7 x 10^7 CFU/ml) of *E. coli* suspension after beads capture.
525 Microtube A3: Magnetic beads bound to *E. coli* cells and separated in a magnetic rack; B. Capturing *M.*
526 *smegmatis* in PBS by beads. Cuvette B1: Concentration (1,68 x 10^7 CFU/ml) of *M. smegmatis* suspension
527 before beads capture; cuvette B2: Concentration (6.56 x 10^5 CFU/ml) of *M. smegmatis* suspension after
528 beads capture; microtube B3: Magnetic beads bound to *M. smegmatis* cells and separated in a magnetic
529 rack.

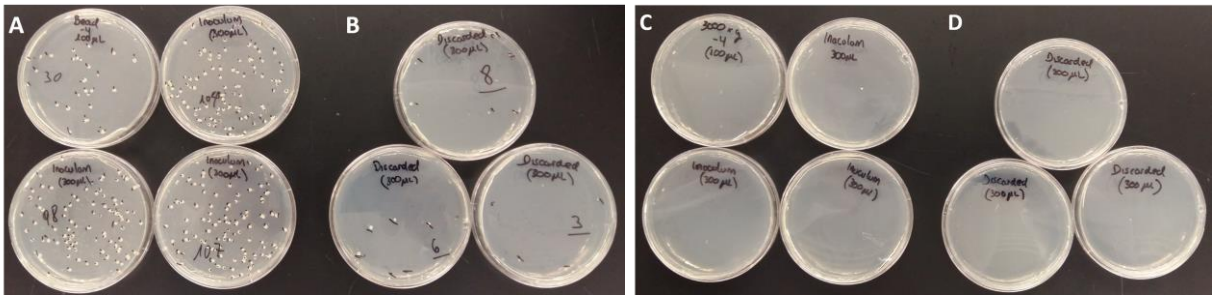


Fig. 2. *M. smegmatis* cells recovered by magnetic beads and centrifugation. From a suspension of 1.68×10^4 CFU/mL of *M. smegmatis*, A and B show cells captured (precipitated) and not captured (supernatant) by beads, respectively. As per the *M. smegmatis* cells remaining in the supernatant (3.7%),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

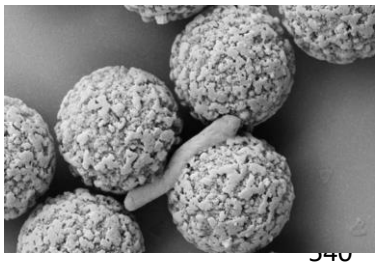
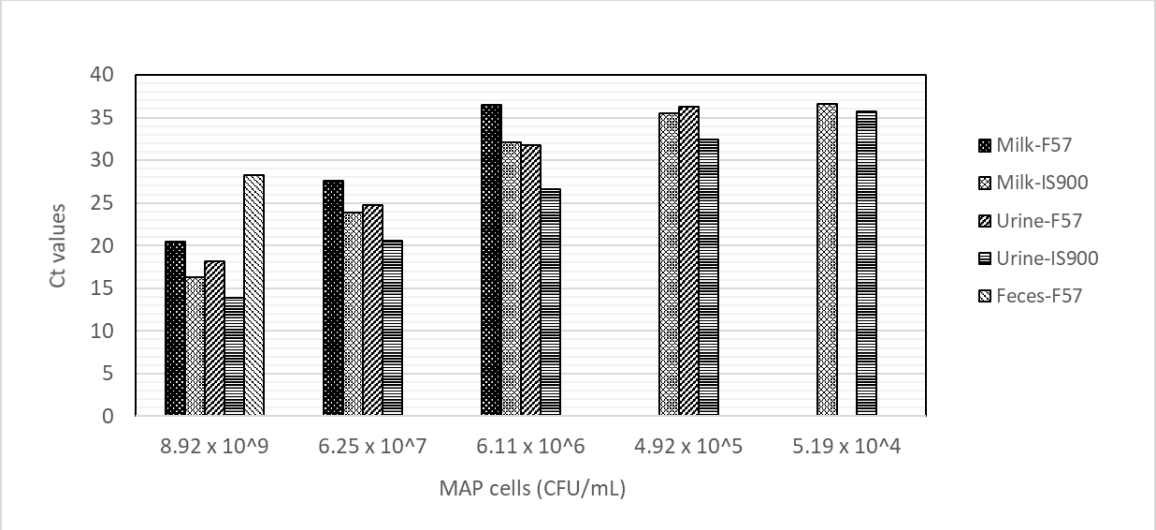


Fig. 3. A scanning electron microscope image of a M. smegmatis cell captured by magnetic beads.

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

544



545

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

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

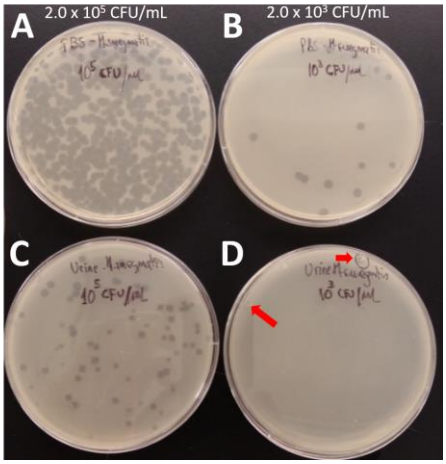
548 and urine, *F57* sequences were detected up to 8.92 x 10^9, 6.11 x 10^6, and 4.92 x 10^5 CFU/mL,

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

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

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

552



553

554 **Fig. 5. Detection of *M. smegmatis* from urine suspensions by mycobacteriophage D29.**

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

556 mycobacteriophage D29 in 24 hours.