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

Gabriel Rojas-Ponce[a*], Dominic Sauvageau[b], Roger Zemp[c], Herman W. Barkema[d], and Stephane Evoy[a]

[a]Department of Electrical and Computer Engineering, University of Alberta, Edmonton, AB, Canada

[b]Department of Chemical and Materials Engineering, University of Alberta, Edmonton, AB, Canada.

[c]Department of Electrical and Computer Engineering, Biomedical Engineering, University of Alberta, Edmonton, AB, Canada.

[d]Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

*Corresponding Author: gabrielr_p@yahoo.com

16 **Abstract**

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

33 **Introduction**

34

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

43 Definitive diagnostic of TB and JD is based on the isolation of MTB and MAP from human and
44 animal's specimens on culture media. For that, the specimen, which can be contaminated with normal
45 flora organisms, should be decontaminated and centrifuged (18). Classical decontamination methods to
46 isolate MTB and MAP on culture method are based on the use *N*-acetyl-L-cysteine–sodium hydroxide
47 (NALC-NaOH) (18) and Hexadecylpyridinium chloride (HPC) (19), respectively, and incorporate the
48 concentration of the specimen at 3000 *x g* at 4°C for 15-20 min (18–20). Given that these chemical
49 decontaminants can kill a proportion of mycobacteria present in the specimen also (19–22), and the
50 availability of a refrigerated centrifuge with sealed safety cups and aerosol tight rotors may be
51 challenging in laboratories from low- and middle-income (23), additional effort should be focused on the
52 development of new and simple methods that enable to separate MTB and MAP without affecting their
53 viability to growth on culture media.

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

61 Tosyl-activated magnetic beads (e.g., Dynabeads M-280 Tosylactivated) can bind covalently to
62 primary amine and sulfhydryl groups in proteins and peptides (37), and as some surface proteins have
63 been identified on mycobacteria (38–40). As per the characteristics of Tosyl-activated magnetic beads,
64 we postulate that Tosyl-activated magnetic beads may interact with these surface proteins for capturing

65 *M. smegmatis* (Ms) and MAP cells in suspension. Because the use of untargeted beads to capture MAP
66 cells would diminish laboratory work, costs, and would increase its affordability in countries with a high
67 burden of TB and JD, this study determined the potential of Tosyl-activated Dynabeads M-280
68 (untargeted magnetic beads) to capture Ms and MAP from spiked urine, feces, and milk samples prior
69 detection by D29 phage and real-time PCR (qPCR).
70
71 **Material and Methods**
72
73 **Bacterial cultures**
74 MAP ATCC 19698 was cultured in Middlebrook 7H9 broth (BD Biosciences, San Jose, CA, USA)
75 supplemented with an oleic acid–albumin–dextrose–catalase mixture (BD Biosciences) and mycobactin J
76 (Allied Monitor, Fayette, MO, USA) at 37°C with constant shaking at 200 rpm for 60 d. Ms mc2155 cells
77 were cultured under the same conditions but without mycobactin and for 7 d. *Escherichia coli* BL21
78 (DE3) was cultured in Luria–Bertani (LB) broth at 37°C for 24 h with shaking. Stock Ms and *E. coli* cells
79 suspensions were adjusted to OD_{600} measurement of 1. Then 4 serial 10-fold dilutions of the stock
80 suspension were made for CFU counting.
81
82 **Magnetic beads assay**
83 Tosyl-activated Dynabeads™ M-280 (Invitrogen)(37) were washed twice with sterile PBS (pH 7.4)
84 before adding into the suspension. Briefly, 21 µl of beads was transferred to one sterile micro centrifuge
85 tubes of 2 ml capacity (microtube) and washed twice with 1 ml of PBS (pH 7.4) for 1 min. Microtube was
86 placed on a magnet (Magna grip rack, Millipore) for removing supernatant. Washed beads were used for
87 magnetic beads assay. Microtubes containing PBS, milk, urine, and feces suspensions spiked with Ms or
88 MAP cells and beads were placed in a microtube rotator (Thermo Scientific™ Labquake™, Edmonton, AB,
89 Canada) at room temperature for 1 h. Then, the microtubes were placed on a magnetic rack for 1 min to
90 carefully remove the supernatant to another microtube. Magnetic beads coupled to Ms and MAP were
91 washed twice with 1 mL PBS before detecting by D29 phage and qPCR.
92
93 **Recovery of viable *E. coli* and *M. smegmatis* by magnetic beads**
94 ***M. smegmatis***
95 From a stock of Ms suspension (adjusted to OD_{600} ~1, microtube#1), 3 serial 10-fold dilutions
96 (microtube#2, microtube#3, and microtube#4) in PBS were made to evaluate the concentration of cells

97 by centrifugation and beads. Determination of viable Ms recovered by beads and centrifugation was
98 estimated as per the number of cells isolated from the supernatant due to the cells sedimented may
99 form clumps and underestimate the count of Ms isolated on LB plates. To make feasible the count of all
100 cells remained in the entire volume of supernatant, the experiments were optimized in microtube (2 mL
101 capacity). Two aliquots (1 mL) of each microtube were transferred to 2 microtubes for centrifugation
102 (control) and beads methods. Microtube#1 (control) was centrifuged at 3000 *x g* at 4°C for 16 min, and
103 the total volume of supernatant was cultured on LB agar plates for counting cells that were not
104 sedimented. Another microtube#1 was assayed for magnetic capture, and the total volume of
105 supernatant was carefully removed and transferred to one plastic cuvette for measuring turbidity via OD
106 at 600 nm and for colony counts method on LB-agar plate. Same procedure, except measuring turbidity,
107 was applied for microtube#2 and microtube#3. From microtube#4, Ms cells recovered by beads and
108 centrifugation were resuspended in 1 mL, vortexed vigorously and plated on LB agar plates. Likewise,
109 the total supernatant volume was plated on LB agar for counting cells. The experiment was repeated 3
110 times.
111 ***E. coli.*** From a stock of *E. coli* suspension (adjusted to OD_{600} ~1), 2 aliquots (1 mL) were transferred to 2
112 microtubes to evaluate the concentration of cells by centrifugation (2000 *x g*, 4°C, 16 min) and capture
113 by magnetic beads. Recovery of cells by centrifugation (control) was done following the procedure
114 described for the microtube#1 with Ms. The experiment was repeated 3 times.
115
116 **Detection of MAP cells by qPCR**
117 This experiment was done to evaluate the recovery of MAP cells by beads and centrifugation
118 through the detection of DNA of the total, viable and non-viable, MAP cells sedimented. Efficiency of
119 beads and centrifugation to capture and sediment cells, respectively, was determined by using 2
120 referential MAP (5×10^7 CFU/ml and 5×10^5 CFU/mL) concentrations. Briefly, 2 serial 10-fold dilutions
121 were prepared from the stock (5×10^7 CFU/mL) of MAP suspension in PBS. Two aliquots (1 mL) of the
122 stock (5×10^7 CFU/ml of MAP cells) were transferred to 2 microtubes to evaluate the recovery of MAP
123 by centrifugation (control) and magnetic beads. Recovered MAP cells by beads and centrifugation (3000
124 *x g*, 4°C, 16 min) were analyzed by F57 qPCR. Same procedure was followed from the second serial
125 dilution containing (5×10^5 CFU/mL MAP). The experiment was repeated three times.
126
127 **Magnetic bead capture MAP from milk, urine and feces**

128 ***From milk and urine suspension.*** Three sterile microtubes containing 0.9 mL of PBS (pH 7.4), 0.9 mL of
129 urine from an apparently healthy human and 0.9 ml of skim milk (Dairyland®) were spiked with 0.1 mL of
130 10^8 CFU/mL MAP cells. Proteinase K (Pk) was added to the microtube containing milk and incubated at
131 70°C for 10 min before magnetic beads assay. MAP cells recovered using beads were analyzed by qPCR.
132 Same procedure was applicable for other microtubes containing less (10^6, 10^5, 10^4 CFU/mL) MAP cells.
133 The experiment was run in triplicate and repeated twice.
134 **From feces suspension.** Cow's feces of a known MAP shedding calf (41) was provided by the University
135 of Calgary. Feces suspension was prepared by mixing 3 g of apparently healthy cattle feces and 6 ml of
136 PBS in a 15-ml centrifuge microtube. After keeping the microtube in an upright position for 20 min, 0.9
137 mL of supernatant was transferred to another microtube and spiked with 0.1 mL of 10^8 CFU/mL MAP cell
138 suspended in PBS. One microtube with 0.9 mL PBS was spiked with 0.1 mL of 10^8 CFU/mL MAP cell. Both
139 microtubes were used for magnetic beads assay and analyzed by F57 qPCR. The experiment was run in
140 triplicate and repeated twice.
141
142 **Quantification of number of MAP cells recovered by beads**
143 MAP cells were counted qPCR based upon the single copy fragment F57 (42). Briefly, DNA
144 calibration curves were prepared for each experiment by preparing serial 10-fold dilutions of DNA
145 sample obtained MAP cell (adjusted to OD_{600} ~1). The initial DNA concentration of the first serial dilution
146 was measured through optical absorbance at 260 nm using a NanoDrop ND 1000 spectrophotometer
147 (NanoDrop Technologies Inc.). DNA molecule copy number (CFU/mL) was determined using the formula:
148 (ng DNA × 6,023 × 10^{23})/(4,829,781 × 10^9 × 660)(43). Number of F57 (CFU) detected in each MAP
149 sediment recovered by beads and control was determined by using the respective threshold cycle (Ct)
150 values.
151
152 **DNA extraction**
153 DNA extraction was performed using a GenoLyse® kit (Hain Lifescience, Nehren, BW, Germany)
154 as per manufacturer's instructions with some modifications. Briefly, MAP cells concentrated by
155 centrifugation or magnetic beads were resuspended in 100 µl of A-Lys, vortexed, centrifuged at 10,000 *x*
156 *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
157 the lysate, vortexed, and centrifuged at 10,000 *x g* for 5 min. Supernatant was transferred to sterile 1.5
158 ml microtubes. Concentration of the isolated DNA was determined by measuring the sample absorbance

159 at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington,
160 DE, USA).
161
162 **Quantitative qPCR assay**
163 This procedure was done as per Singh et al. (36). Briefly, qPCR amplification targeting IS900 and
164 F57 genes was done using Taqman specific probes and StepOnePlus Real Time PCR System (Applied
165 Biosystems). The 10 µL PCR mixture comprised 5 µL of TaqMan® Universal Master Mix II (Applied
166 Biosystems) with the uracil-N-glycosylase (UNG), 200 nM of each primer and 250 nM of fluorogenic
167 probe specific to IS900 and F57. From each template DNA sample, 4 µL was added in the reaction
168 mixture. qPCR conditions were as follows: 50°C for 2 min for UNG treatment, initial denaturation at 95°C
169 for 10 min, 40 cycles at 95°C for 15 sec, and at 60°C for 1 min. Each qPCR amplification targeting IS900
170 and F57 genes was run in triplicate with negative and positive controls. Obtained data were analyzed in
171 the form of threshold cycle (Ct) values.
172
173 **Magnetic separation of *M. smegmatis* from urine for mycobacteriophage D29 infection**
174 The experimental procedure was adjusted as per infection of MTB by D29 phage (44) and
175 doubling time (3 to 4 hours) of Ms (45). Two urine aliquots of 1 ml of 2.0 x 10^5 and 2.0 x 10^3 CFU/mL Ms
176 were transferred to 2 sterile microtubes. Ms captured by beads from urine were detected by D29 phage
177 (44,46) with some modifications. Briefly, Ms cells captured by beads were resuspended in 150 µL of LB
178 with 1 mM $CaCl_2$ (LB-S) and infected with 50 µL of 10^8 PFU/mL D29 phage at 37°C. Extracellular D29
179 phage was eliminated with 0.03 M ferrous ammonium sulfate (FAS) for 10 min; then 0.5 mL of LB-$CaCl_2$
180 was added to the microtube for stopping FAS virucide activity. The total volume suspension was mixed
181 with 4 mL of sterile molten agar (0.8%) and 1 mL of 10^8 CFU/mL Ms suspension before pouring onto
182 Petri dishes with LB agar. Petri dishes were incubated for 24 h at 37°C and the number of infection
183 plaques was counted. The experiment was repeated 3 times.
184
185 **Statistical analyses**
186 Number of F57 and IS900 copies, determined in triplicate per each sediment recovered by u-
187 TDbeads and control, were plotted and analyzed by the Kruskal-Wallis test (GraphPad Instat, version 8;
188 GraphPad Prism, La Jolla, CA). *P*-values < 0.05 were considered statistically significant.
189
190 **Results**

191

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

193 Magnetic beads captured Ms cells in PBS with greater capture efficiency compared to *E. coli* cells
194 (P <0.05). This is evident by the lack of cells in supernatant of cuvette B2, unlike cuvette A2, where
195 beads efficiently removed cells from suspension (Fig. 1). The number of viable Ms cells isolated from the
196 supernatant of each suspension showed higher (> 90%) recovery of viable cells by beads than
197 centrifugation. *E. coli* cells were not recovered by beads; approximately 94% of cells from suspension
198 were isolated from the supernatant (Table 1). The effect of the centrifugation and magnetic beads
199 methods on the viability of Ms cells was confirmed from the suspension 1.68 x 10^4 CFU/mL (Fig. 2). As
200 per one experiment results, 17 CFU of Ms isolated from the supernatant and 337 CFU-beads recovered
201 from the sediment of the suspension (1.68 x 10^4 CFU/mL), 337 CFU compressed 1326 CFU of Ms as
202 consequence of the formation of clumps during the magnetic separation (Fig. 2). Thus, the magnetic
203 beads recovered more (96%) viable Ms from the suspension (1.68 x 10^4 CFU/mL) than the centrifugation
204 (0%). An electron microscopy image of the capture of Ms cells by beads is presented in Fig. 3.

205

206 **Detection of MAP cells by qPCR**

207 The initial concentration (100%) of MAP cells in the first and second PBS suspensions was
208 estimated by detecting F57 sequence from all MAP cells collected by centrifugation. Since the
209 centrifugation of PBS suspensions was done at 3,000 *x g* at 4°C for 16 min to concentrate 95%
210 mycobacteria (18), we estimated that the initial concentration (100%) of MAP cells in the first and
211 second PBS were 2.93 x 10^9 and 1.43 x 10^7 CFU/mL. Using F57 qPCR, there was a difference (p=0.04) in
212 recovery of MAP cells: from the first suspension 2.8 x 10^9 CFU/mL (Ct = 17.89) were recovered by
213 magnetic beads and 3.2 x 10^9 CFU/mL (Ct 17.68) by centrifugation, whereas 3.9 x 10^7 CFU/mL (Ct =
214 23.94) were recovered by magnetic beads and 1.4 x 10^7 CFU/mL (Ct = 25.43) by centrifugation
215 ((p<0.0001); Table 2).

216

217 ***Milk, urine, and feces suspension.*** Ct values demonstrated that magnetic beads captured no less than
218 10^9 CFU/mL from feces and not less than 10^4 CFU/mL of MAP cells from milk and urine suspensions (Fig.
219 4). From milk suspensions the recovery rates, confirmed by F57 sequences, were 6.11% of 8.92 x 10^9
220 CFUL/mL, 5.96% of 6.25 x 10^7 CFU/mL, and 0.62% of 3.80 x 10^4 CFU/mL. Pk was incorporated in milk
221 suspensions as our previous experiments (data not shown) showed that its activity increased the
222 capture of MAP cells by beads. Recovery rates of MAP cells from urine suspensions using F57 was

223 30.93% of 8.92 x 10^9, 43.25% of 6.25 x 10^7, 44.56% of 6.11 x 10^6 CFU/mL, and 8.60% of 4.92 x 10^5
224 CFU/mL. IS900, unlike F57, was more sensitive to detect lowest (10^4 CFU/mL) MAP cells concentration
225 from milk and urine (Fig. 4 and Table 3). From feces, 0.66% of 8.92 x 10^9 CFU/mL MAP cells were
226 captured and detected by beads and F57-qPCR (IS900 was not done). Other feces suspensions
227 containing lower concentrations were not detected.
228
229 **Magnetic separation of *M. smegmatis* from urine for mycobacteriophage D29 infection**
230 Magnetic beads-D29 phage detected Ms spiked in urine in 24 h (Fig. 5). Although diminished
231 number of plaques were observed from urine spiked with 2 x 10^5 CFU/mL and 2 x 10^3 CFU/mL of Ms
232 (Table 4), the number of PFU detected from urine suspension with 2 x 10^5 CFU/mL of Ms clearly prove
233 the presence of Ms in the suspension in 24 h.
234
235 **Discussion**
236
237 Untargeted Tosyl-activated Dynabeads™ M-280 recovered more (>90%) viable Ms from spiked PBS
238 suspension with 10^7, 10^6, 10^5 and 10^4 CFU/mL Ms. Capture efficiency was estimated by counting
239 unbound Ms cells (supernatant) that were not captured nor sedimented by beads. Counting cells
240 sedimented by beads, and even, after resuspending them in 1 mL before plating (47), could
241 underestimate the real number of cells recovered by beads due to formation of Ms and MAP clumps
242 during the sedimentation process. With regards to the effect of magnetic beads and centrifugation on
243 the viability of Ms cells, untargeted magnetic beads did not affect the viability of Ms to growth on LB
244 agar plate and to be infected by D29 phage. Contrary, centrifugation had a negative effect on the
245 viability of Ms cells sedimented from all four 4 suspensions spiked with 4 different Ms concentrations.
246 Moreover, the qPCR detection of MAP cells, after sedimentation by centrifugation and magnetic beads,
247 indicate that the use of magnetic beads would be the best method to precipitate cells from PBS
248 suspension. Although the centrifugation method (3000 *x g* at 4°C for 16 min) has been established as a
249 referential method to recovery 95% of cells from suspension (18,48), we agree with others (49–51) that
250 centrifugation may not be an appropriate method to concentrate specimens containing small numbers
251 of organisms. The inefficient centrifuge method may be related to the growth rate of bacteria; low
252 recovery of MAP cells from a stationary culture is expected as the majority of cells have a lower buoyant
253 density due to the presence of lipid bodies (52). Likewise, as others have reported the effect of

254 centrifugation process for other bacteria (53), the low number of Ms growth on LB agar may be
255 explained by the damage of some bacterial cells, and even DNA, during the centrifugation.
256 Since detection of MAP cells from milk improved after treating with Pk, we postulate that Tosyl
257 group can be blocked by milk proteins and, as consequence, affect the capture of MAP cells. The effect
258 of Pk on capture of MAP cells from milk was not observed in urine nor feces, perhaps, due to the higher
259 protein concentration (1.87%) of milk (54) compared to urine (55) and feces. With regards to the
260 magnetic separation of MAP cells from urine and feces, we postulate that these differences in the
261 detection sensitivity of qPCR to detect MAP cells from milk, urine and feces can be explained by its own
262 inhibitors, and the number of copies of F57 (1 copy) (56,57) and IS900 (14-20 copies)(57,58) in the
263 genome of MAP. Urea amine group, and other inhibitors such as polysaccharides originated from
264 vegetable material in the diet (59), gut flora, bile salts, and glucolipids (60), may react with Tosyl groups
265 and decrease the capture of cells by beads. Likewise, these inhibitors of each specimen could be
266 transferred with the beads coupled to MAP cells and reduce the amplification efficiency of F57 and
267 IS900 sequences.
268 Magnetic beads detected viable Ms from urine suspension containing at least 10^3 CFU/mL by
269 D29 phage. Since that our experiments have shown that the centrifugation process can affect the
270 viability of all Ms cells from a suspension with 10^6 CFU/mL bacilli, and considering that the
271 decontamination of sample may damage the mycobacteria and reduce the sensitivity of phage-based
272 assays for detecting mycobacteria cells (61), our method magnetic bead-based D29 phage could also be
273 useful to detect low amount of MAP cells from cow's milk, and even MTB from urine samples, in 24 h.
274 However, since the number of plaques (02 PFU) from urine suspension spiked with 2 x 10^3 CFU/mL of Ms
275 may raise questionable results, the addition of an incubation step of recovered Ms at 37°C for 24 hours,
276 before adding phages, could be necessary to increase the number of cells to be infected by phages, and
277 as consequence, the increase of plaques units on the indicator plate. Despite that the final
278 concentration of phages (2.5 x 10^7 PFU/mL) in the PBS and urine suspensions should ensure an infection
279 rate of 1% (maximum infection rate) (44), the presence of beads in PBS and, as well, the urine urea
280 would explain the low infection rate (<1%) of cells by phage. Beads may interfere the interaction
281 between cells and phages while the urine urea (amino group) may interact with Tosyl groups and reduce
282 the number of cells captured by beads.
283 Another variable that positively contributed to the capture of mycobacteria by beads was
284 related to the technical skills to take aliquots of beads in stock suspension, remove the supernatant after
285 capturing beads coupled to mycobacteria and washing beads. Before taking an aliquot of the stock

286 beads suspension, it was homogenized by pipetting 3 – 4 times carefully. The supernatant was removed
287 without taking out the microtube from the magnetic rack to avoid removing beads coupled to
288 mycobacteria. PBS washing process was done carefully despite that, due to the characteristic of beads
289 for interacting with proteins, there would be a strong interaction between beads and mycobacteria
290 surface proteins. PBS was added slowly and carefully through the inner microtube's wall and not directly
291 to the beads. The microtube should be slightly inverted 5 times to ensure that all beads distributed
292 homogenously and are not making visible clumps that can carry on some debris present in the sample.
293 The use of microcentrifuge tube of 2 mL with attached flat top cap ensures a) homogenous mix of
294 suspension for increasing the attraction between beads and cells, b) removal of supernatant while the
295 tube is placed in the magnetic rack, and c) addition of PBS to the tube for washing cells coupled to
296 beads.
297 Notwithstanding others (62) demonstrated that the use of Tosyl-activated Dynabeads™ M-280
298 coupled to Gp10, a recombinant lysin from the mycobacteriophage L5, in combination with qPCR, the
299 study does not show data about the capture efficiency of uncovered magnetic beads. Another study (47)
300 showed that other types of magnetic beads coupled to some peptides and antibodies, and
301 complemented with phages, detected around 0.03% and 0.003% of 10^3 to 10^4 CFU/mL MAP cells from
302 milk, respectively. However, unlike the method of this study, which recovered MAP cells directly from
303 milk, the MAP cells were sedimented from milk by centrifugation and then were transferred to one
304 microtube containing 7H9-OADC media for magnetic separation of MAP cells. Moreover, the authors
305 (47) showed that other types of uncoated beads recovered a moderate percentage (around 10%) of
306 nontarget mycobacteria.
307 A limitation of the study was that MAP cells recovered by beads were not cultured. Although the
308 2 log10 lower counts of MAP cells between theoretical concentration determined at OD600 and F57-
309 qPCR can be explained by the clumps present in the suspension and not detected at OD600, and our
310 results are in line with another study (42) that have also reported a difference of two log10 counts
311 between colony counts on culture media and F57-qPCR, counting these recovered cells before qPCR
312 could have been useful to infer the presence of death and alive cells recovered by beads and
313 centrifugation. Other variable that could not be controlled in the study were the effect of calcium
314 chelating agent in milk. Removing calcium from milk could reduce the formation of MAP cells clumps
315 and keep a homogenous distribution of cells in the suspension to be captured by beads. This study did
316 not evaluate the effect of less amount of feces (1 g (19,63), 2g (64,65)) on the capture of MAP cells; even
317 though less amount of feces can provide less amount of inhibitors, the reduction of feces would reduce

318 the sensitivity to recovery MAP cells. The evaluation of these variables in further studies would define
319 the potential to use untargeted magnetic beads to capture MAP cells from milk and feces.
320 The results of this study encourage further effort to evaluate the utility of Dynabeads® M-280
321 Tosylactivated, without specific ligand, in real animals' samples with a presumptive diagnosis
322 of paratuberculosis. Likewise, the use of these beads, in combination with a microbiological (e.g., DS6A
323 phage, culture media, colorimetric method) or a molecular test (e.g., HAIN-MTBDRplus, HAIN-MTBDRsl,
324 GeneXpert), would have high potential to be used as a simple and rapid tool for diagnosis of TB disease.
325
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Table 1: *Escherichia coli* and *Mycobacterium smegmatis* remained in supernatant after centrifugation and magnetic beads 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, $\pm 1.22 \times 10^2$)

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]

[a] Approximation of CFU in each suspension was assessed quickly by OD600 measurements.

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

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

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

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

Initial concentration (CFU/mL)	Milk				Urine				Feces		
	F57-qPCR			IS900-qPCR	F57-qPCR			IS900-qPCR	F57-qPCR		
	CFU/mL (mean, ±SD)	Recovery (%)	Ct (mean; ±SD)	Ct (mean; SD)	CFU/mL (mean, ±SD)	Recovery (%)	Ct (mean; ±SD)	Ct (mean; ±SD)	CFU/mL (mean, ±SD)	Recovery (%)	Ct (mean; ±SD)
8.92×10^9	5.45×10^8; ± 8.31×10^7	6.11	20.45; ± 0.22	16.30; ± 0.18	2.76×10^9; ± 4.46×10^8	30.93	18.12; ± 0.23	13.84; ± 0.08	5.93×10^7 ± 1.54×10^7	0.66	28.27; ± 0.35
6.25×10^7	3.73×10^6; ± 1.01×10^6	5.96	27.61; ± 0.38	23.89; ± 0.13	2.70×10^7; ± 6.85×10^6	43.25	24.77; ± 0.34	20.53; ± 0.06	UND	UND	UND
6.11×10^6	3.80×10^4 ; ± 2.44×10^4	0.62	36.44; ± 1.11	32.14; ± 1.74	2.72×10^6 ; ± 2.81×10^6	44.56	31.72; ± 2.71	26.63; ± 2.93	UND	UND	UND
4.92×10^5	UND	NA	NA	35.45; ± 0.78	4.23×10^4 ; ± 2.70×10^4	8.60	36.24; ± 0.82	32.42; ± 2.01	UND	UND	UND
5.19×10^4	UND	NA	NA	36.59; NA	UND	NA	NA	35.74; ± 1.18	UND	UND	UND

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

	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

*Mean of three experiments.

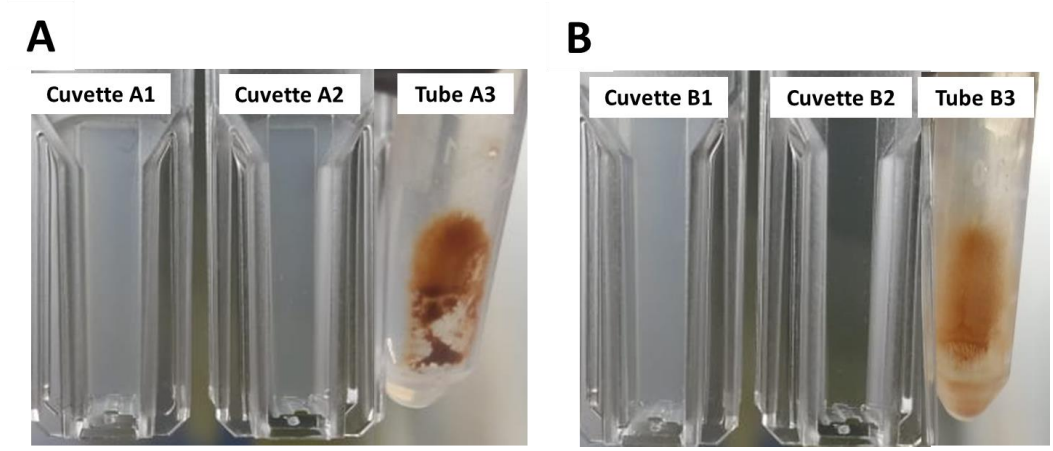

FIG 1 Capture of *E. coli* and *M. smegmatis* cells in PBS suspension by magnetic beads.

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

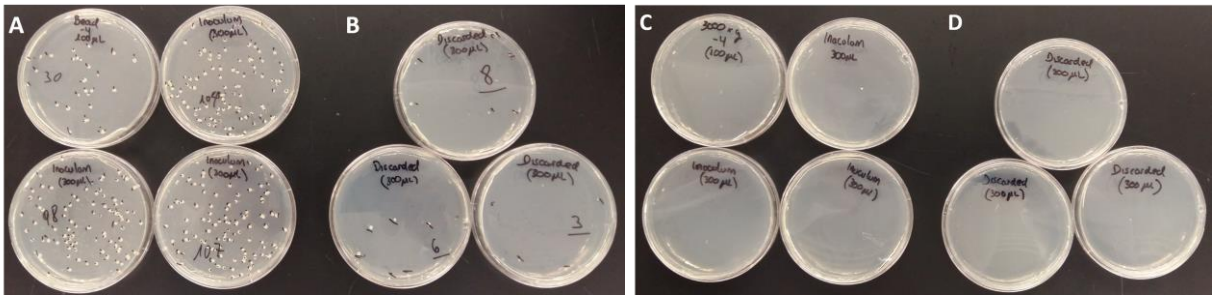

FIG 2 *M. smegmatis* cells recovered by magnetic beads and centrifugation. From a suspension of 1.68 x 10^4 CFU/mL of *M. smegmatis*, A and B show cells captured (precipitated) and not captured (supernatant) by beads, respectively. As per the recovery (3.69%) of *M. smegmatis* cells from the supernatant, the estimated number of cells recovered was 96.31%. C and D show that there were not cells recovered nor remained in the supernatant after centrifugation process.

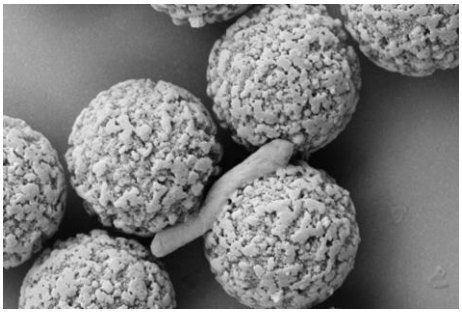

FIG 3 Electron microscopy of *M. smegmatis* cell captured by magnetic beads. Capturing *M. smegmatis* cell in PBS by magnetic beads.

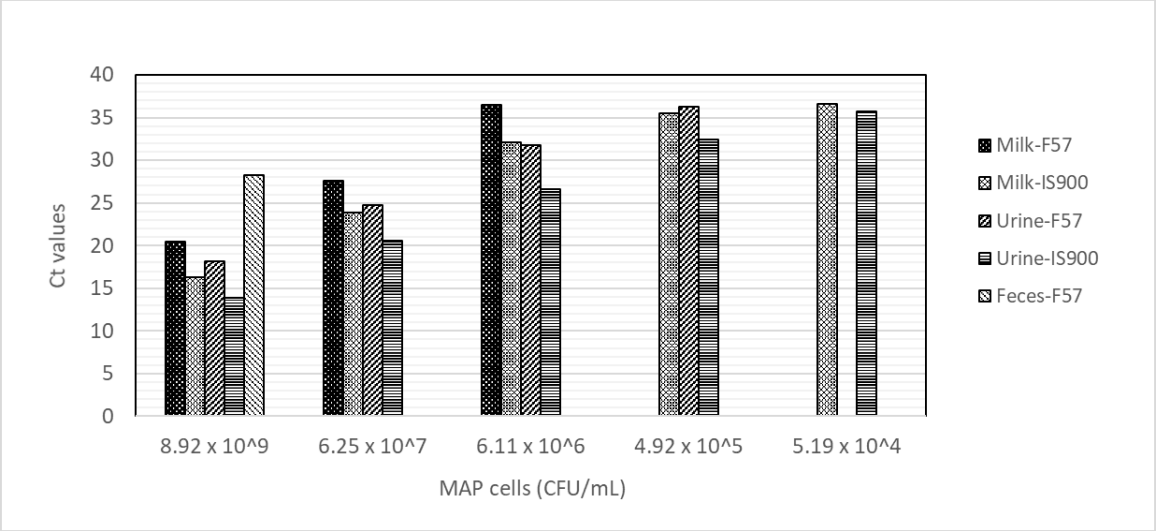

FIG 4 Detection of the F57 sequence by qPCR from MAP cells recovered by magnetic beads.

Ct values showed that magnetic beads captured MAP cells from milk, urine, and feces. From feces, milk, 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, respectively. IS900, unlike F57, showed better sensitivity to detect lowest (10^4 CFU/mL) MAP cells concentration from milk and urine. Other feces suspensions containing lower concentrations were not detected by qPCR. Detection of IS sequence from feces sample was not done.

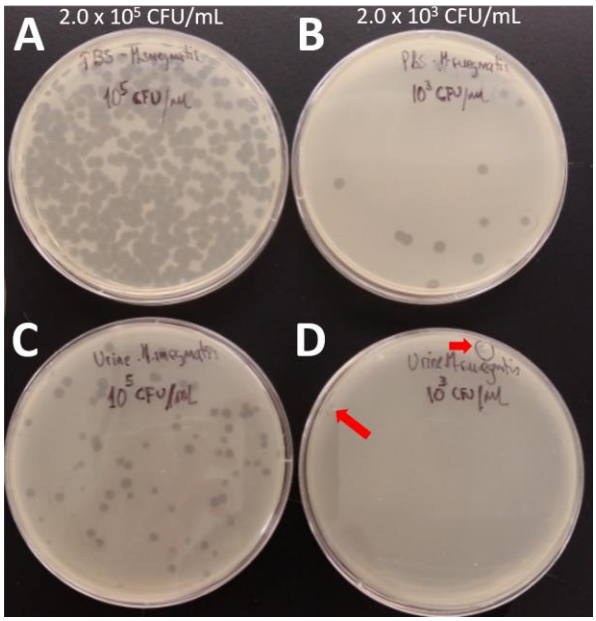

FIG 5 Detection of *M. smegmatis* from urine suspensions by mycobacteriophage D29.

Detection of *M. smegmatis* captured from PBS suspensions (A and B) and urine suspensions (C and D) by mycobacteriophage D29 in 24 hours.