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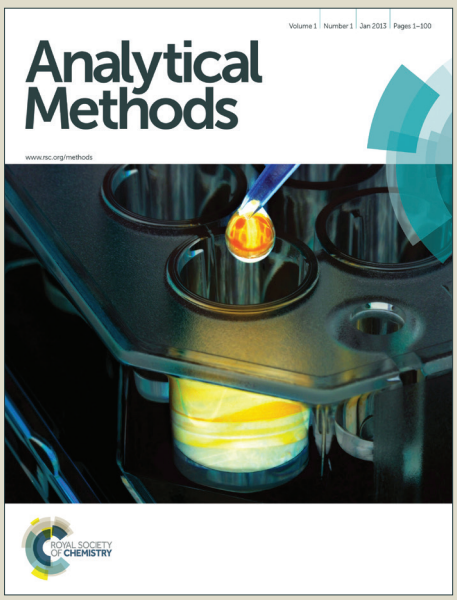

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Mycobacteriophage lysin-mediated capture of cells for the PCR detection of *Mycobacterium avium* subspecies *paratuberculosis*

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Abstract

Recombinant lysin Gp10 from the mycobacteriophage L5 was coupled to magnetic Dynabeads 280 and these beads were used to capture *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cells from complex media. Skim cow milk spiked with MAP cells, skim milk spiked with both MAP and *Escherichia coli* cells and Middlebrook 7H9 medium spiked with MAP cells were used to model the contaminated food matrices. The beads bearing the immobilized Gp10 were incubated with the samples, separated, washed, subjected to DNA extraction and the solution was analyzed by real time PCR. The entire process was completed within 24 hours, demonstrated high specificity towards the MAP cells and increased the sensitivity of detection. The C_t values observed from the pre-concentrated samples were close to those obtained from clean buffer, whereas they were significantly worse without such bead-based pre-concentration. The protocol was successfully tested with two MAP strains (ATCC 19698 and 19851) and two target sequences (IS900 and F57). The suggested method eliminates the need of lengthy culturing steps used in traditional protocols and allows the pre-concentration of MAP cells to get rid of the various PCR inhibitors that may be present in the food matrices. As such, it offers an overall reduction of the time usually required to test milk for MAP infections. The developed protocol may be instrumental for the prevention, diagnostics and monitoring of Johne's and Crohn's diseases in cattle and humans, respectively – two gastrointestinal diseases having huge economic and public health impact.

Key words: Johne's disease**,** Crohn's disease, mycobacterium, PCR, phage, lysin, magnetic separation.

Introduction

Foodborne diseases continue to cause a high level of morbidity and mortality, specifically for infants, young children, elderly and immunocompromised individuals (1). The magnitude of the problem remains underrated due to inaccurate reporting in many parts of the world. Contaminants can occur in food at any stage of processing. It may result from environmental contamination, pollution from water, soil or air and from production to consumption (2). Development of innovative strategies for food and livestock monitoring are thus necessary.

More specifically, it is estimated that one out of ten cattle sold in auction facilities is infected by Johne's disease (3). This gastrointestinal disease leads to the dramatic loss of the productivity and the eventual death of the animal resulting in significant economic losses. Johne's disease causes a loss between $ 200 million to $ 250 million annually to the American dairy industry (3). In 2007, a National Animal Health Monitoring Systems (NAHMS) study showed that 68.1 % of U.S. dairy cattle are infected with *Mycobacterium avium* subsp. *paratuberculosi*s (MAP), the causative organism of Johne's disease (3). About one out of four U.S. cattle operations includes a relatively high percentage of MAP-infected cows in its herds (3). This pathogen possesses a unique persistence in the intestine and the infected animal may not display symptoms of the disease for years (4). One cow is thus sufficient to infect 50% of the herd before any symptom is observed (3). The infected animals shed MAP in their milk and feces contributing to the rapid dissemination of infection among the industrial herds and in the environment. Thus, the human population may also be exposed to MAP through consumption of the contaminated retail milk and ground water. Apparent survivability of this pathogen in retail pasteurized milk remains a matter of particular concern (5) taking into account that almost every patient diagnosed with Crohne's disease was infected with MAP (6). A relationship between a chronic inflammatory bowel disease in humans and MAP seems to exist (7, 8). In 2002, it was estimated that between 400 000 to 600 000 patients in North America suffered from this disease and were intensively but not always successfully treated with corticosteroids, 5-aminosalicylate products and surgery (6).

The detection of MAP in human and animal tissues remains challenging. The pathogen has developed extraordinary resistance to chemical and enzymatic lysis. Lack of an optimized mechanical disruption step in sample processing hinders early diagnostics of MAP infections. *M.*

avium subsp. *paratuberculosi*s has primarily been detected through culturing of fecal samples. Culturing of MAP cells from fecal samples followed by polymerase chain reaction (PCR) based analysis has recently emerged as the new golden standard for the diagnosis of this pathogen (4). However, it takes 25 days to obtain a positive test result due to the extremely slow growth rate of this mycobacterium. Furthermore, a conclusively negative test result can require more than two months of incubation. PCR is also somewhat strenuous in complex samples such as fecal material and dairy food due to the presence of the natural inhibiting agents (5). Alternative immunological assays are impeded by antibody degradation, cross reactivity and variability during different stages of infection and are often incapable of detecting subclinical MAP infection. FASTPlaque TB bacteriophage propagation based assays in combination with PCR were also suggested for the detection of MAP (9). Yet, this approach remains indirect and will inevitably be dependent on the quality of the live phage preparations used in the assay – a parameter which was always difficult to standardize (10). Robotization of this assay may be problematic, too (9). Recent studies have reported the use of PCR for the detection of MAP in milk (11, 12, 13). These studies have however outlined some of the limitations of the technique. For instance, while these assays have used IS900 was used as a template, it is apparent that this template is not suitable for accurate quantification (14). Another study have reported the combination of MGIT media culture system was with real time PCR for the confirmation of MAP cells as well as for the replacement of radioactive BACTEC 460 assay system (15). Such approach however required 7 weeks of incubation prior to confirmation by real time PCR.

We previously demonstrated the use of immobilized bacteriophage particles for the detection of *Escherichia coli* (16). A recent review article has also indicated an enormous potential of bacteriophages for the rapid detection of pathogenic bacteria (17). This being said, our work concluded that recombinant phage receptor binding proteins (RBPs), responsible for the phage-host specificity, can be used as biological probes and present numerous advantages over the use of whole phage particles. We have reported the use of RBPs as novel probes for the capture of several foodborne pathogenic bacteria (18, 19, 20, 21). Phage RBPs immobilized onto magnetic particles were successfully used for rapid isolation (in less than 3 hours) of bacterial cells from food samples spiked with *Campylobacter jejuni* cells (22).

Lysins represent another class of cell envelope binding phage proteins that degrade the host cell walls and promote the release of newly formed phage particles (34). Engineered lysins have been suggested as reagents for the rapid detection of listeria and staphylococci (35, 36). We demonstrated that the immobilized recombinant lysin Gp10 from the mycobacteriophage L5 (27, 28, 37) was able to capture MAP cells from saline solutions (38). We are currently reporting the use of recombinant Gp10 for the capture of MAPs from complex natural matrices. The recognition of cells by Gp10 was leveraged as a pre-concentration step for the rapid and specific extraction of mycobacteria followed by a validated real time PCR technique. In contrast to prior reports involving other pathogens (22), a comparative assessment of capture involving different subspecies has been performed. The present study is also innovative in its use of two target sequences (IS900 and F57) for the PCR analysis. Indeed, a number of PCR based assays have been reported for the detection of MAP in milk and the IS900 insertion element is an established standard marker for the detection of MAP (23). Specificity of the PCR assays based on this target sequence is however impeded by the presence of highly homologous sequences in other mycobacteria. A few alternative target elements such as F57, IS Mav2 and HspX have been evaluated (24, 25, 26). While the F57 element is more specific for MAP, it is also known to show lower levels of amplification than IS900. That is why two target sequences were used in our study and the effectiveness of the Gp10 mediated capture of MAP cells was conclusively established.

Materials and Methods

Materials

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), monosodium phosphate, sodium phosphate dibasic, and ammonium sulfate were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) solution was prepared by mixing one BupH phosphate buffered saline pack (Pierce) with 500 ml of MilliQ-grade water (Millipore) yielding a solution of 0.1 M sodium phosphate and 0.15 M NaCl (pH 7.2).

The detailed procedure for Gp10 production will be described in a separate publication. Briefly, gene 10 was amplified using a suspension of mycobacteriophage L5 obtained from the Felix D'Herelle Reference Centre for Bacterial Viruses (Laval University, Quebec, Canada) and cloned into a pET30a vector (Novagen). The His-tagged recombinant protein (approximately 40

kDa polypeptide) was produced in *E. coli* BL21 (DE3) cells and purified using immobilized metal affinity chromatography. The Gp10 preparation was dialyzed against PBS and used in the subsequent experiments. The protein concentration was determined by measuring the absorbance at 280 nm and assuming $A_{0.1\%}$ of 1.402 for the His-tagged Gp10. The ProtParam Tool (www.expasy.org) was used to calculate the extinction coefficient (53400 M^{-1} cm^{-1}). The A_{280}/A_{260} ratio was approximately 1.8 for the Gp10 preparation.

Bacterial strains and culture media

The bacterial cultures used were *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19851, *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698, *Mycobacterium marinum* ATCC 927, *Mycobacterium smegmatis* mc^2155 and *Escherichia coli* BL21 (DE3). The mycobacteria strains were cultured in Middlebrook 7H9 broth (BD Biosciences, USA) supplemented with an oleic acid-albumin-dextrose-catalase mixture (BD Biosciences, USA) and mycobactin J (Allied Monitor, USA). All these cultures were incubated at 37^{o}C with constant shaking at 200 rpm for 10 days. *M. smegmatis* cells were grown under the same conditions but without mycobactin and were incubated for only 48 hours. *Mycobacterium marinum* ATCC 927 was also grown without mycobactin but incubated for 12 days in the same growth conditions as described above. All mycobacterial cells were sonicated for 5 min using the Branson Ultrasonics 1510 sonicator (40 kHz frequency, 80 W power) to make a homogeneous cell suspension prior to any subsequent manipulations. *E. coli* BL21 (DE3) was cultured in Luria-Bertani broth overnight at 37 ^{o}C with shaking.

Immobilization of Gp10 onto Tosylactivated Dynabeads® M-280

M-280 Tosyl-activated Dynabeads® were purchased from Life Technologies Inc. (USA). These beads are uniform magnetic round particles having a diameter of 2.8 μm, coated with a polyurethane layer and activated with *p*-toluensulfonyl chloride. The primary amino groups of the proteins react covalently with the sulfonyl ester group present on the beads (27, 29).

Twenty microliters of 100 mg/ml Dynabeads® M-280 were washed twice with sterile PBS for 10 min. The tubes containing washed beads were placed on a magnet for 1 min, and the supernatant was removed. The beads were resuspended in 1 ml of sterile PBS. The cleaned beads were

incubated with 100 µg/ml Gp10 for 1 h at 37°C followed by overnight incubation at room temperature. The functionalized beads were further incubated with 1 mg/ml BSA for 30 min to block the free surface and prevent the nonspecific binding. All the incubations were done with gentle shaking. Finally, the beads were washed twice with sterile PBS to remove any unbound BSA.

Capture of bacterial cells from milk and other media

Skim milk powder (BD Biosciences) was suspended in PBS to 1-3 % (w/v) concentration and artificially spiked with $2.4x10^7$ cfu/ml (0.25 OD_{600}) *M. avium* subsp. *paratuberculosi*s ATCC 19851 or *M. avium* subsp. *paratuberculosi*s ATCC 19698. In another experiment, the suspension of skimmed milk in PBS was artificially spiked with $2.4x10^7$ cfu/ml of MAP cells and $2.4X10^7$ cfu/ml of *E. coli* BL21(DE3) cells. *M. marinum* ATCC 927 and *M. smegmatis* mc^2155 were also used to spike the milk in the same way. Since mycobacterial cells aggregate into clumps due to their hydrophobic cell surfaces (30), the cells were sonicated for 5 min prior to incubation with the magnetic beads to get a homogeneous suspension and to prevent non-specific deposition of aggregated cell masses onto the bead surfaces. The Gp10 functionalized beads were suspended in the spiked milk or in sterile PBS buffer spiked with the MAP cells as per the protocol described above, and were incubated for 1 h at room temperature under gentle shaking. The beads were separated by incubating the samples on a magnetic separator for 5 min and washed twice with sterile PBS. The Gp10 functionalized beads were also used to capture bacteria directly from Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase and mycobactin J. Fully grown cultures of *M. avium* subsp. *paratuberculosi*s ATCC 19851 and *M. avium* subsp. *paratuberculosi*s ATCC 19698 were diluted to 0.25 OD_{600} ($2.4X10^7$ cfu/ml) and exposed to Gp10 functionalized beads for 1 h at room temperature in the same way as described above. The beads were then separated from the medium, washed and analyzed.

Fluorescence microscopy

Once exposed to bacteria, the beads were washed with PBS and exposed to 50 µM resazurin (Sigma-Aldrich) in DMSO for 20 min. The stained beads were then washed twice in PBS to remove the excess dye. An Olympus IX81 microscope equipped with a FITC filter and a Roper Scientific Cool-Snaps HQ CCD camera were used to record the fluorescence images.

Extraction of genomic DNA

Genomic DNA was extracted from mycobacteria using the QuickExtract DNA extraction kit (Epicenter Biotechnologies) according to the manufacturer's instructions with some modifications. The samples were incubated for 1 hour with 50 µl of QuickExtract DNA extraction solution and 2 µl of Ready – Lyse lysozyme solution. This process was followed by incubation at 80^{o}C for 1 hour in a water bath for complete lysis of the cells. The concentration of the isolated DNA was determined by measuring the sample absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.).

Primers and probes for the target sequences IS900 and F57

The primers and probes were purchased from Integrated DNA Technologies. Primers and the TaqMan probe (Applied Biosystems) were designed using Primer Express 3.0 software to amplify the fragments of the sequences IS900 and F57 found in all *M. avium* subsp. *paratuberculosis* cells (Table 1). Forward and reverse primers containing the TaqMan probe were selected. The probe was labeled with the reporter dye 6-carboxyl-flurescein (FAM) at the 5'end and with the non-fluorescent Black Hole Quencher Dye (BHQ) at the 3' end.

Table 1. Primers and probes used in the study.

Primers/Probes	**Target sequence IS900**	**Target sequence F57**
Forward Primer	*5'- TGACGGTTACGGAGGTGGTT -3'*	*5'-CGGTCCAGTTCGCTGTCAT-3'*
Reverse Primer	*5'-ATGCAGTAATGGTCGGCCTTA-'3*	*5'-CACGCAGGCATTCCAAGTC-3'*
Taqman Probe	*5'-TGGCACAACCTGTCTG-3'*	*5'-ACGGGAAGGGTGGTC-3'*

Quantitative real time PCR assay

The quantitative real time PCR amplification was performed using Taqman specific probes and StepOnePlus Real Time PCR System (Applied Biosystems). Each reaction mixture (10 µl volume) comprised of 5 µl of TaqMan® Universal Master Mix II (Applied Biosystems) with the uracil-N-glycosylase (UNG), 200 nM of each primers and 250 nM of fluorogenic probe. Four µl of each template sample was added in the reaction mixture. The real time PCR cycling conditions were as follows: 50°C for two min for UNG treatment, 95°C for 10 min to denature the UNG and activate the DNA polymerase, 40 cycles of 15 s at 95 ºC and 1 min at 60 °C. Each data point was run in triplicate with negative and positive controls. The obtained data was analyzed in the form of threshold cycle (C_t) values (Tables 2 and 3), where the target amplification is first detected at which fluorescence intensity is greater than background fluorescence.

Table 2. Recovery rates of *M. avium* subsp. *paratuberculosis* ATCC 19851 by the Gp10 functionalized magnetic beads and the Ct values of the different samples. "ND" – not determined.

Serial number	Sample type	Real time PCR (Ct) Target gene IS900	% Recovery	Real time PCR (Ct) Target gene F57	% recovery
1.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19851 Preconcentrated from PBS	15.5 ± 0.18	85.5 %	19.9 ± 0.27	80.4 %
2.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19851 Preconcentrated from 1 % milk	15.9 ± 0.012	81.76 %	20.6 ± 0.15	77.6 %
3.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19851 Preconcentrated from 2 % milk	16.7 ± 0.14	77.8 %	21.5 ± 0.22	74.41 %
4.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19851 Preconcentrated from 3 % milk	16.5 ± 0.083	78.78 %	21.3 ± 0.31	75.11 %
5.	*Mixture of E.coli and Mycobacterium avium subsp. paratuberculosis* ATCC 19851 Preconcentrated from 3 % milk	17.3 ± 0.049	75.14 %	22.15 ± 0.1	72.22 %
6.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19851 in 3 % milk (not concentrated)	23.5 ± 0.29	55.3 %	35.7 ± 0.27	44.81 %
7.	*Mycobacterium smegmatis mc2155* Preconcentrated from 3 % milk	23.3 ± 0.059	ND	28.2 ± 0.10	ND

Table 3. Recovery rates of *M. avium* subsp. *paratuberculosis* ATCC 19698 by the Gp10 functionalized magnetic beads and the Ct values of the different samples. "ND" – not determined.

Serial number	Sample type	Real time PCR (Ct) Target gene IS900	% Recovery	Real time PCR (Ct) Target gene F57	% Recovery
1.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19698 Preconcentrated from PBS	13.9 ± 0.044	93.53 %	18.9 ± 0.33	84.64 %
2.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19698 Preconcentrated from 1 % milk	13.7 ± 0.18	94.8 %	18.6 ± 0.31	86.02 %
3.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19698 Preconcentrated from 2 % milk	13.8 ± 0.14	94.2 %	18.4 ± 0.30	86.9 %
4.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19698 Preconcentrated from 3 % milk	13..9 ± 0.035	93.5 %	18.8 ± 0.25	85.1 %
5.	*Mixture of E.coli and Mycobacterium avium subsp. paratuberculosis* ATCC 19698 Preconcentrated from 3 % milk	13.6 ± 0.17	95.5 %	17.1± 0.2	93.5 %
6.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19698 in 3 % milk (not concentrated)	22.08 ± 0.30	58.87 %	29.8 ± 0.4	53.69 %
7.	*Mycobacterium marinium ATCC 927* Preconcentrated from PBS	*30.6* ± 0.22	ND	35.8 ± 0.25	ND
8.	*Mycobacterium avium subsp. paratuberculosis* ATCC 19698 Preconcentrated from Middlebrook 7H9 *media*	13.9 ± 0.195	93.5 %	17.1 ± 0.02	93.5 %

Preparation of the standard amplification curves

Standard amplification curves were generated in order to quantify the DNA extracted from the mycobacterial cells. A set of standard DNA solutions was generated by preparing serial 10-fold dilutions of DNA obtained from a 2.4 x 10^7 cfu/ml cell suspension. The DNA concentration of these serial dilutions was independently measured through optical absorbance at 260 nm using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc.).

Calculation of the recovery rate

The recovery rate (*RR*) was calculated using the following equation:

$$RR(\%) = 100 \times \frac{Ct_0}{Ct_c}$$

Ct_0 is the maximal theoretical Ct value corresponding to the total amount of DNA in the aliquot of the spiked sample. This value was calculated on the basis of the standard curve obtained for the pure DNA samples in PBS. Ct_c is the Ct value obtained for the DNA preparation obtained from the washed beads used to pre-concentrate the cells from the aliquot of the spiked sample. Standard curves were made for each experiment and the recovery rates were calculated from the respective standard curves.

Results and Discussion

Capture of mycobacterial cells by the Gp10 functionalized Dynabeads

The fluorescence microscopy results showed that *M. avium* subsp. *paratuberculosi*s ATCC 19851, *M. avium* subsp. *paratuberculosi*s ATCC 19698 and *M. smegmatis* mc^2155 (Figure 1 B – D, respectively) cells were captured by the Gp10 functionalized magnetic beads. The magnetic beads were moderately auto-fluorescent and were easily detected in the field of view as was previously described (21). One can see the bright and "clustered" fluorescence areas on the bead surface representing the bound MAP cells. In addition to that, a strong aggregation of the beads was noted after exposure to the MAP cells but not in the control experiment. This effect was similar to what we observed when *C.jejuni* cells were pre-concentrated using beads covered with *C.jejuni* phage RBP (21) and we believe occurs due to several bacterial cells binding simultaneously to more than one bead.

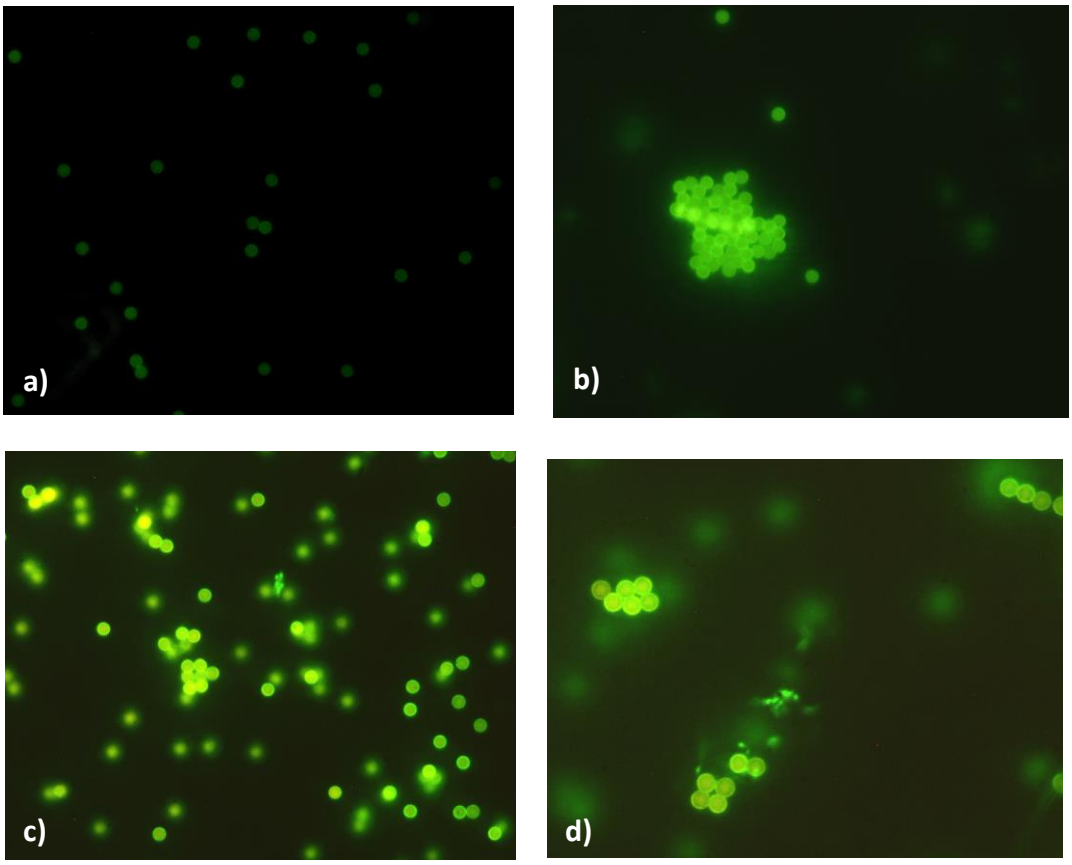

Figure 1. Fluorescent images of bacterial capture by GP-10 RBP derivatized magnetic beads. All pictures were recorded at 100X magnification A) the beads were not functionalized by GP-10 RBP. No bacterial capture was observed in the sample B) Capture of *Mycobacterium avium subsp. Paratuberculosis ATCC number 19851. Bacterial capture was observed. C) Capture of Mycobacterium avium subsp. Paratuberculosis ATCC number 19698 Bacterial capture was observed. D) Capture of Mycobacterium smegmatis mc215. Bacterial capture was observed.*

Thus, no capture of *E. coli* BL21 (DE3) cells was observed (Figure 2). Interestingly, no significant capture of *M. marinum* ATVV 927 was observed. It was concluded that the immobilized Gp10 can be efficiently used to capture mycobacterial cells with some degree of specificity across species. Yet, an additional step was needed to distinguish between MAP and *M. smegmatis* cells extracted by the functionalized beads.

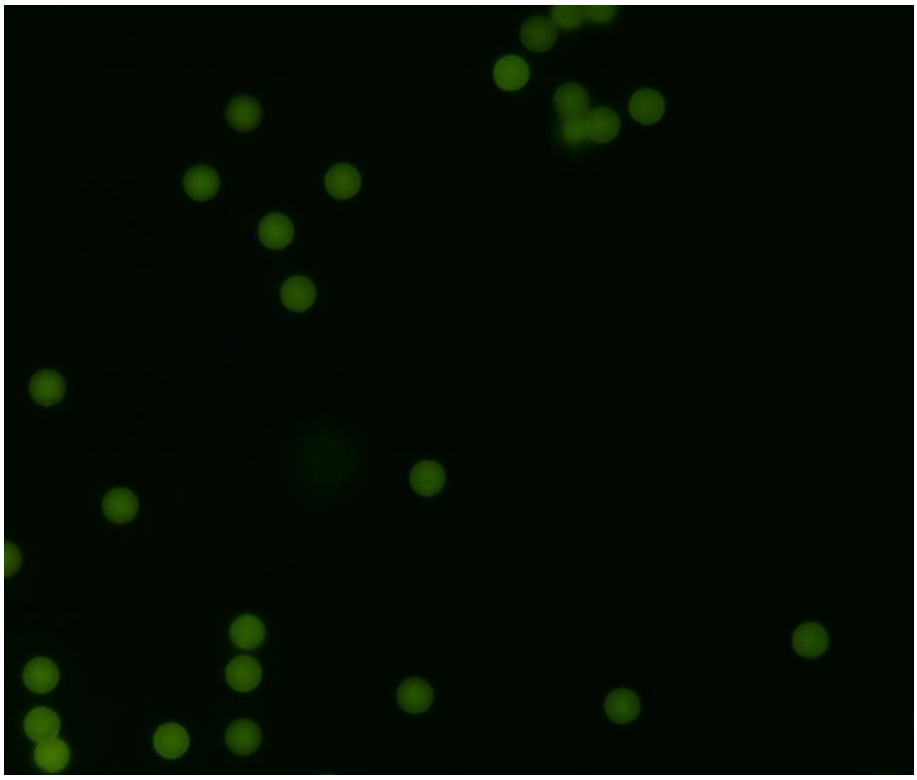

Figure 2. Fluorescent images of *E.coli BL21 (DE-3)* capture by GP-10 RBP derivatized magnetic beads. No bacterial capture was observed indicating the specificity of the Protein. Pictures were recorded at 100X magnification.

Specific detection of MAP in artificially contaminated milk

The real time PCR assay was evaluated using a known concentration of MAP cells. Two types of target sequences, F57 and IS900, were used to validate the specificity as well as the sensitivity of the assay. The standard curves of the real time PCR analysis maintained a linear character for up to six orders of magnitude of the cellular concentration, i.e. from $2.4X10^{1}$ cfu/ml to $2.4X10^{7}$ cfu/ml. These standard curves showed a R^2 value of 0.99 and slope of -3.295 for the F57 sequence (Figure 3). For the IS900 target, the standard curve showed similar characteristics with a R^2 value of 0.99 and a slope of -3.282 (Figure 4). No increase in fluorescence was observed above the base level in the reagent control (no template) samples.

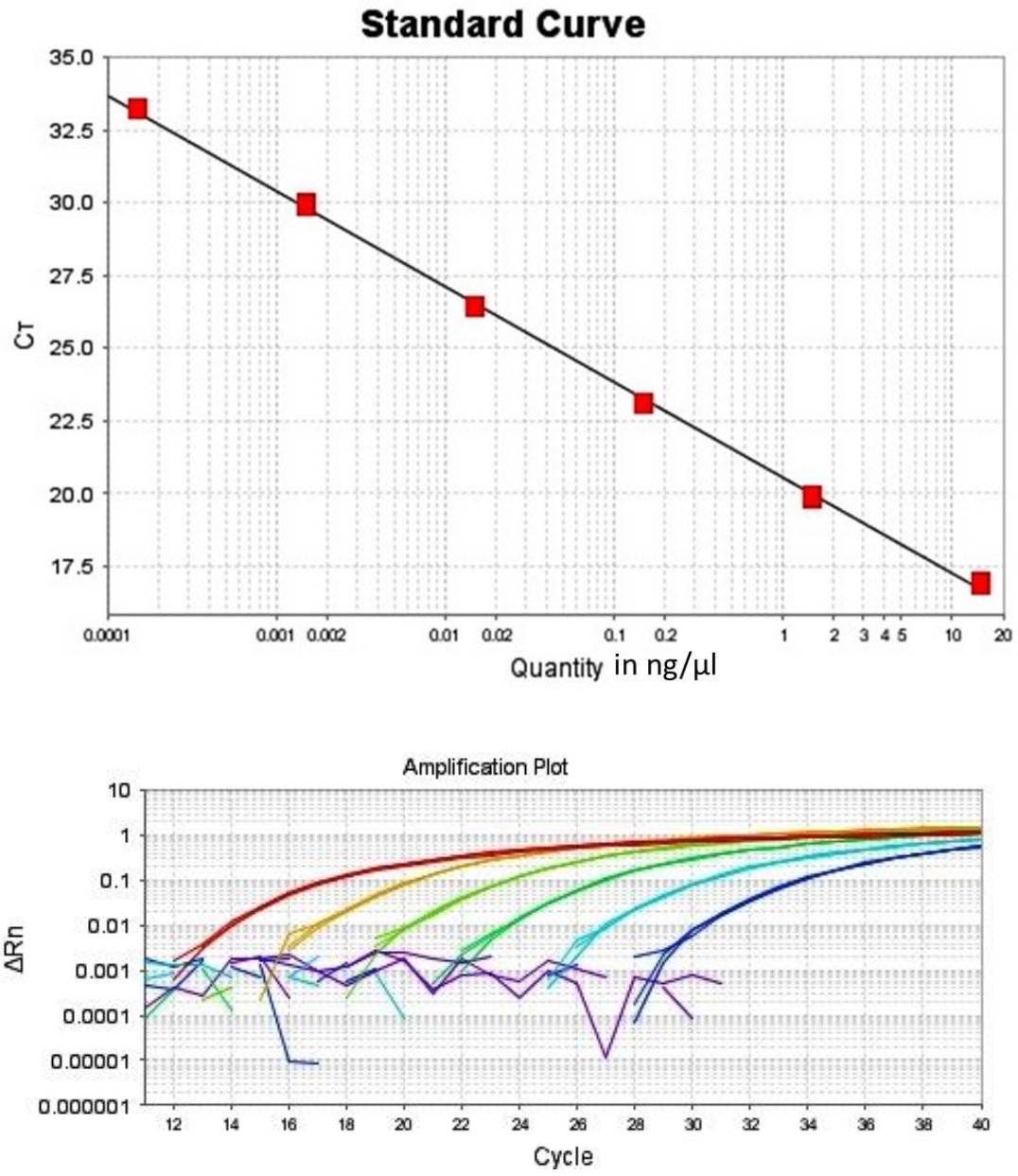

Figure 3. Real time PCR based detection of the mycobacterial target sequence F57. A) Standard DNA amplification curve for the 10-fold dilution series of *M. avium* subsp. *paratuberculosis* ATCC 19851 starting from highest concentration of 15 ng/µl. Standard curve was generated from the correlation of the threshold cycle values (Ct) with the DNA concentration of *M. avium subsp. paratuberculosis* ATCC 19851 cells in each dilution (ng/µl). Each dot represents the results of four data point amplifications for each dilution. The slope of the regression curve is -3.295 and the R^2 = 0.99. B) Amplification plot for the standard curve shown in panel A.

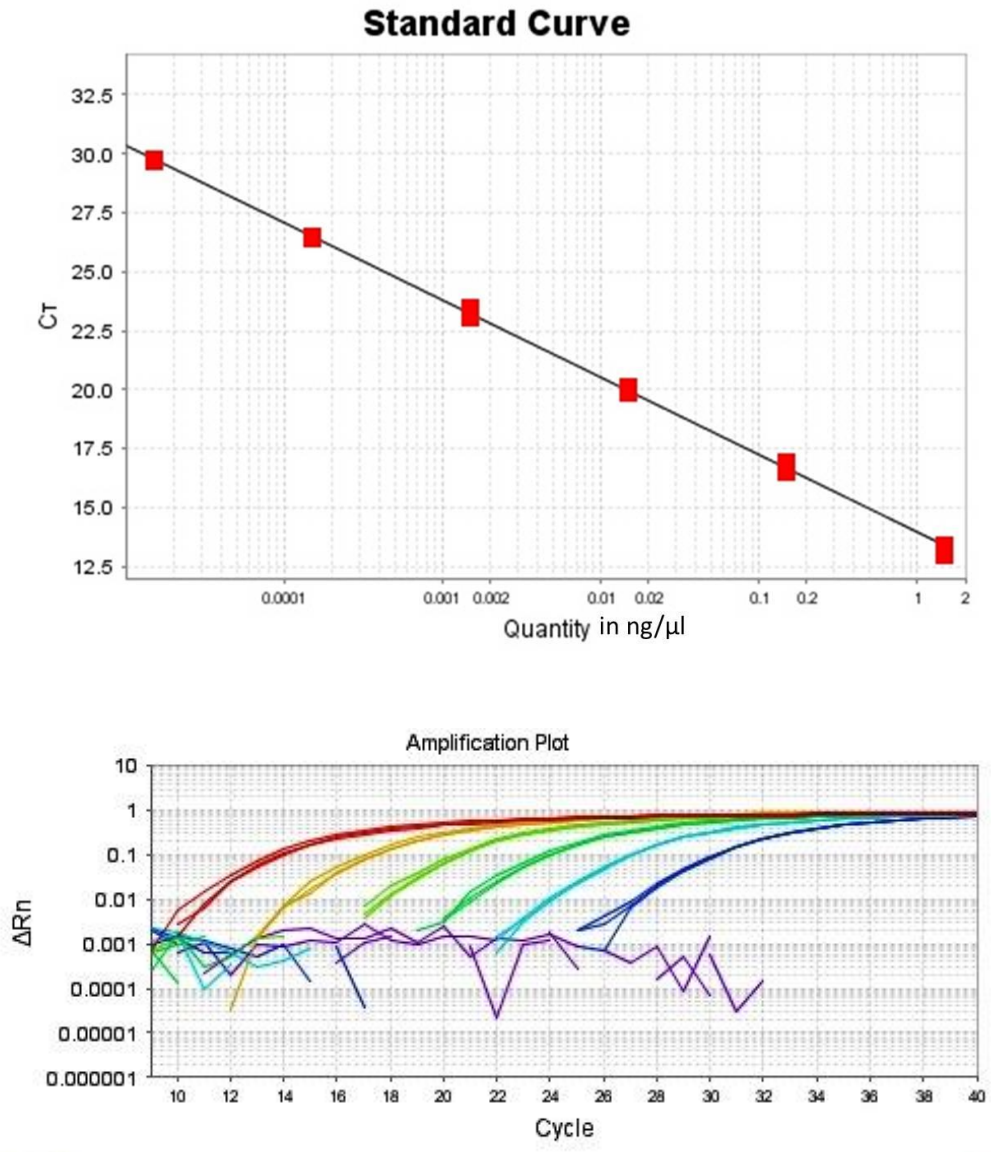

Figure 4. Real time PCR based detection of the mycobacterial target sequence IS900. **A)** Standard DNA amplification curve for the 10-fold dilution series of *M. avium* subsp. *paratuberculosis* ATCC 19851 starting from highest concentration of 1.5 ng/µl. Standard curve was generated from the correlation of the threshold cycle values (Ct) with the DNA concentration of *M. avium subsp. paratuberculosis* ATCC 19851 cells in each dilution (ng/µl). Each dot represents the results of four data point amplifications for each dilution. The slope of the regression curve is -3.282 and the R^2 = 0.99. **B)** Amplification plot for the standard curve shown on panel A.

The Lysin-Dynabead based cell pre-concentration method was coupled with real time PCR to quantify MAP in milk spiked with MAP cells. The Ct values turned out to be the lowest for the pre-concentrated samples (Tables 2 and 3). *M. marinum* ATCC 927, *M. smegmatis* mc^2155 and *E. coli* BL21 (DE3) were used as references to check the specificity of the method. One can conclude that MAP cells can be easily pulled out from a complex environment contaminated with other bacteria. Also, MAP cells can be easily discriminated from the other mycobacteria when the suggested two-step protocol is used. Notably, in all cases the recovery rates of the samples pre-concentrated from the milk were close to those of the PBS-based samples whereas the recovery rates of the non-concentrated milk samples were considerably lower. This result indicates that the described protocol increases the sensitivity of the PCR based detection of MAP cells in milk.

Comparison between the Ct values of the two target sequences

There are 14-20 copies of IS900 per MAP genome, which allows a high level of sensitivity for the PCR based detection. However, the specificity of IS900 became questionable when a DNA sample from slow growing non-pathogenic environmental *Mycobacterium cookii* showed strong amplification (31). Considering this limitation of IS900, other target sequences have been suggested (32-33). We tested one of them, the F57 sequence, in combination with our pre-concentration protocol. The greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct.

The highest recovery rate was 85% when MAP cells were pre-concentrated and 55% when the cells were not pre-concentrated and the target sequence IS900 was used (Table 2). The highest recovery rate was 80 % for the pre-concentrated MAP cells and 44 % when the cells were not pre-concentrated and the target sequence F57 was used (Table 2).

Similar experiments were done for the other strain of MAP (Table 3). Again, the highest recovery rate was 95 % for the pre-concentrated cells and 58 % for the non pre-concentrated cells when the IS900 sequence was used. Again, the highest recovery rate was 93 % when the cells were pre-concentrated and 53% for the non pre-concentrated cells when the F57 sequence was targeted. Additional negative control experiments where the functionalized beads were intermixed with matrices spiked with *E. coli* BL21(DE3) The captured material, if any, did not

show any onset of PCR amplification of the related target gene, confirming that no significant capture of that pathogen was performed by the beads. Thus, our pre-concentration protocol can be successfully applied for PCR analysis where several target sequences are used as an additional safeguard to ensure reliable discrimination between MAP and other mycobacteria.

Conclusions

The traditional and most reliable method for MAP detection is cell culture combined with PCR. However, MAP is a slow growing bacterium that takes 25 days to grow in expensive media. In the current study, we demonstrate that MAP cells can be pre-concentrated using an immobilized phage lysine. This can then be followed by PCR based analysis resulting in a rapid, highly specific, sensitive and robust process of MAP cell detection. Results suggest that MAP cells were effectively extracted from artificially contaminated milk with some level of discrimination from other species of mycobacteria. A full assessment of specificity would however require a systematic study across a large number of other subspecies. Remarkably, the long culturing step was eliminated and a validated real time PCR approach could still be used to achieve the clinically approved golden standard of detection. The developed protocol has a potential for being transformed into an automated high throughput format. A study examining feces samples of MAP infected cows is under progress. Similar kinds of studies can be also being done to create diagnostics for Crohn's disease in humans. Also, the described method can potentially be applied for the detection of other *M. avium* subspecies. Indeed, *M. avium* is a serious human pathogen causing extensive lung damage and disseminated infections in immunocompromised individuals and cystic fibrosis patients (39). Novel tools for the monitoring of these infections are also urgently needed.

Acknowledgements

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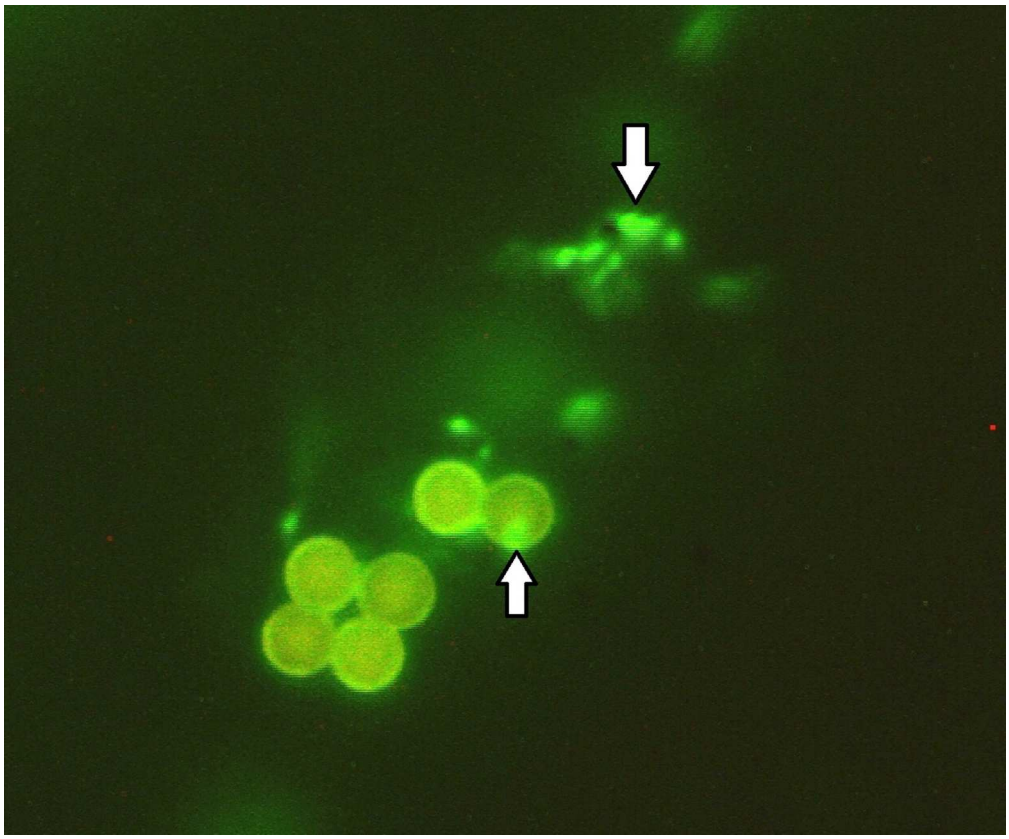

M. avium paratuberculosis cells captured by beads functionalized with phage-derived lysin
210x174mm (150 x 150 DPI)