Comparison of Two Immunomagnetic Separation Methods for Detection of *Mycobacterium avium* subsp. *Paratuberculosis* in Bovine Feces

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ABSTRACT

Detection of *Mycobacterium paratuber culosis* in feces is complicated by the slow-division and the lipid composition of the cell wall of the bacterium. In this study, we compared a rapid and sensitive method for direct detection of *M. paratuberculosis* with anti-*paratuberculosis* chicken IgY-conjugated magnetic nanoparticles versus commercially available micro-sized paramagnetic beads. Tenfold serial dilutions of a standard *M. paratuberculosis* strain and clinical isolation strains were spiked in pooled stool and detected by using antibody-conjugated magnetic nanoparticles or paramagnetic beads, and confirmed by polymerase chain reaction. The binding ratio of antibody-nanoparticles was 96.44 ± 0.77%, and the detection limit was 20 *M. paratuberculosis* cells per gram of feces. Also, magnetic nanoparticles showed significantly intensive results than paramagnetic beads in dose-effects evaluation. The magnetic nanoparticles were also cost-effective. Antibody-conjugated magnetic nanoparticles did not react with other bacteria, thus providing specific immunomagnetic separation. We conclude that the antibody-conjugated magnetic nanoparticles are more effective than paramagnetic beads for low-cost, direct detection of *M. paratuberculosis* in cattle feces; in addition, confirmation time was reduced from 12−16 weeks to 2−3 days. This antibody-based immunocapture polymerase chain reaction method is expected to provide a platform of rapid screening of *M. paratuberculosis* in fecal samples in one step.

INTRODUCTION

Paratuberculosis or Johne's disease (JD) is a type of chronic enteritis in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This leads to diarrhea, nutrient malabsorption, reduced milk production, and emaciation. This pathogen has also been implicated as a possible cause of Crohn's disease in humans. Crohn's disease is a chronic inflammatory bowel disease that can occur in any part of the gastrointestinal tract.[1]

Detection of MAP in feces is complicated by its long subclinical phase, intermittent pathogen shedding in low numbers, polymerase chain reaction (PCR) inhibitors in the feces, and cell wall lipid composition. The gold standard method for MAP diagnosis is culture-based,[2, 3] but this method requires 12−16 weeks of incubation time and sensitivity varies by clinical disease stage; sensitivity is 91% in clinical stages and 45% to 72% in subclinical stages.[4] The bacterial culture also requires at least 100 CFU g^{-1} feces; thus, infection may not be detected in animals with early infection stage.[4] Enzyme-linked immunosorbent assay (ELISA) is the most widely used screening test for bovine paratuberculosis. ELISA provides very good sensitivity and specificity in clinically ill animals, but animals in the initial and terminal stages yeild false-negative results due to anergy.[5] PCR is widely used for disease detection because of its high sensitivity, specificity, and rapid turn-around time. To identify MAP, IS900 is unique and has been used widely as a specific and sensitive DNA marker.[6] However, inhibitory factors may be isolated with the target nucleic acid and reduce PCR performance.[7] This limitation is often associated with failure of the DNA extraction method to completely remove inhibitors in the sample.[8] Therefore, new DNA extraction methods removing inhibiting substances have been needed.

Immunomagnetic separation (IMS) is used to detect organisms in various matrices. IMS can be used to diagnose animals in early and terminal stages of infection because it targets surface antigens.[9, 10] IMS applications also include detection of viruses, parasites, and bacteria.[11-13] Previous studies have adopted antibody-conjugated magnetic particles with culture-based methods, ELISA, and PCR to increase the sensitivity of MAP detection.[14-16]

The aim of this study was to evaluate a

new procedure to specifically isolated MAP from fecal samples using antibody-conjugated magnetic nanoparticles followed by IS900-based PCR assay.

MATERIALS AND METHODS

Chemicals and bacterial strains

Media, an antibiotic mixture, and Middlebrook oleic acid-albumin-dextrose-catalase (OADC) were from BD Biosciences (Franklin Lakes, NJ, USA). Mycobactin J was from Allied Monitor (Fayette, MO, USA). Magnetic nanoparticles (MNPs, SPM-NH2) were provided by Nanobrick (Gyeonggi-do, Korea) and paramagnetic beads (PMBs) were purchased from Life Technologies (Dynabeads®, Carlsbad, CA, USA). The bicinchoninic acid protein assay kit was from Pierce (Rockland, IL, USA) and PCR premix was from Bioneer (AccuPower™ PCR HotStart PreMix, Daejeon, Korea). All other materials were from Sigma (St. Louis, MO, USA).

We used MAP ATCC 19698 (American Type Culture Collection, Manassas, VA, USA) as a standard strain and four clinically isolated MAP strains from cattle were also used. The MAP strains were cultured on modified Middlebrook 7H10 medium supplemented with 0.1% casitone, 0.5% glycerol, an antibiotic mixture (10,000 U polymyxin B, 1 mg amphotericin B, 4 mg nalidixic acid, 1 mg trimethoprim and 1 mg azlocillin; Panta Plus, BD Biosciences), 10% (v/v) Middlebrook OADC, 2 μg/mL Mycobactin J and egg yolk of specific-pathogen-free eggs (added 250 mL to 1 L of medium). An antibiotic mixture, OADC, egg yolk, and Mycobactin J were added after autoclaving. MAP strains were incubated at 37°C for 8 weeks. Strain stocks were prepared and stored at −80°C as 1-mL aliquots in Middlebrook 7H9 broth containing 15% glycerol.

To examine specificity, we used *Mycobacterium* species including *M. bovis* ATCC 35726 and clinical isolations of *M. intracellulare*, *M. fortuitum*, *M. smegmatis*. Mycobacteria were grown on Middlebrook 7H10 medium containing 10% (v/v) OADC enrichment with (for *M. intracellulare*, *M. fortuitum* and *M. smegmatis*) or without (for *M. bovis*) 0.5% glycerol. These strains were incubated at 37°C for 8 weeks. In addition, gram-positive bacilli such as *Bacillus subtilis* KCTC 2023 and *B. sporothermodurans* KACC 10906 were used. *Bacillus* species were inoculated on brain heart infusion agar and incubated at 37°C for overnight.

Preparation of antibody-conjugated MNPs or PMBs

MNPs used in this study were silica-coated and surface-modified with an amino-group via silanization with aminosilane. Scanning electron microscopy (SEM) was used to examine MNPs shape. Dynamic light scattering (DLS) was measured with a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) to determine the size distribution.

Polyclonal anti-MAP chicken IgY was produced as described and kindly provided by Dr. Shin.[10] Four 24-week-old White Leghorn chickens were used for IgY production. Antibody was conjugated with MNPs via glutaraldehyde coupling. Briefly, 2 mg MNPs were suspended in coupling buffer (0.01 M pyridine, pH 6) and were separated using a magnet. This process was repeated twice. Separated MNPs were activated in 5% glutaraldehyde by incubation for 30 min at room temperature. Next, MNPs were magnetically separated and washed three times in coupling buffer. About 100 μg antibody was suspended in coupling buffer; 100 μl of this solution was used as the pre-coupling solution. Antibody in coupling buffer was mixed with 2 mg MNPs and reacted at room temperature for 16−24 h with gentle agitation. MNPs were separated using a magnet; the supernatant was used as a post-coupling solution. Separated MNPs were re-suspended in glycine quenching solution (1 M glycine, pH 8) and then allowed to react at room temperature for 30 min with gentle agitation. After quenching, MNPs were magnetically separated and washed three times in coupling buffer. These samples were resuspended in storage buffer (0.01 M Tris, 0.15 M NaCl, 0.1% bovine serum albu-

Table 1. *Zeta-potential of magnetic nanoparticles*

			Mean (mV)	Area (%)	Width (mV)
Zeta-potential (mV)	32.1	Peak 1:	32.1	100.0	5.58
Zeta-deviation (mV)	5.58	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm)	0.00174	Peak 3:	0.00	0.0	0.00

min, pH 7.4) and stored at 4°C. The protein concentration of antibody-conjugated MNPs was calculated by estimating the pre- and post-coupling solution by bicinchoninic acid protein assay. Coupling buffer was used as a blank.

Commercially available paramagnetic beads (PMBs), Dynabeads® M-280 Streptavidin were used for comparison. Dynabeads® are micro-sized, uniform, paramagnetic beads composed of polystyrene (2.8-µm diameter) activated by streptavidin covalently attached to the surface. Antibody-PMBs conjugation was performed according to manufacturer instructions.

Bacterial detection using antibody-conjugated MNPs or PMBs

To prepare artificially contaminated samples, bacterial cells were harvested and suspended in 10 mL phosphate-buffered saline (PBS), then vigorously vortexed for 3 min to disperse large clumps. This suspension was used to estimate MAP cell number and to perform tenfold serial dilution. An optical density of 1.00 at 600 nm was determined to equal 10^8 cells/mL as described by Shin et al.[17] To prepare the pooled stool solution, five bovine fecal samples previously identified as culture- and PCR-negative for MAP were collected and mixed; 1 g of mixed stool was suspended in 20 mL PBS.

The sensitivity of antibody-conjugated MNPs or PMBs was investigated as described by Chui et al.[18] with some modification. First, MAP ATCC 19698 and four clinical isolation strains (two each from cattle feces and intestine) were serially diluted (10^5, 10^4, 10^3, 10^2, 10^1 and 1) and spiked into PBS or pooled stool solution. Samples were centrifuged at 8,000 rpm for 2 min and the supernatant was discarded. The samples were resuspended in PBS with 6% sodium dodecyl sulfate (SDS) and shaken at 150 rpm for 30 min. Cells were pelleted by centrifugation at 8,000 rpm for 2 min. The pellet was washed twice in PBS (pH 7.4) and resuspended in 990 µL PBS. After adding 10 µL antibody-conjugated MNPs or PMBs (antibody final concentration, 1µg), the reaction tubes were incubated at 37°C for 1 h with shaking at 150 rpm. After incubation, the samples were placed on a magnetic separator for 10 min, after which the supernatant was discarded. Samples were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and isolated by standing in magnetic separator for 5 min. Samples were resuspended in 100 µL TE buffer and placed on a 100°C heat block for 15 min to lyse the cells. The samples were centrifuged at 13,000 rpm for 5 min; the supernatant was used as a template for PCR.

PCR primers and protocol were performed according to the method described by Moss et al.[19] with some modifications.

Table 2. *Amount of anti-paratuberculosis chicken* IgY1

	In pre-coupling solution (µg mL^{-1})	In post-coupling solution (µg mL^{-1})	Binding ratio (%)[2]
Anti-*paratuberculosis* chicken IgY	103.36 ± 1.25	3.68 ± 0.28	96.44 ± 0.23

1 Data represent mean ± SD.

2 Binding ratio: ((Pre-coupling – Post-coupling)/ Pre-coupling) × 100.

Primers were designated P90 (5′-gtt cgg ggc cgt cgc tta gg-3′) and P91 (5′-gag gtc gat cgc cca cgt ga-3′) targeting the insertion element IS900 of MAP. Each primer (10 pmol) was added to the PCR mixture, which included PCR premix (1 U Taq DNA polymerase, 250 μM each dNTPs, reaction buffer containing 1.5 mM $MgCl_2$; AccuPower™ PCR HotStart PreMix, Bioneer) and 2 μL genomic DNA. PCR amplification was conducted in a PCR machine (Mastercycler, Eppendorf Mastercycler, Hamburg, Germany) using the following protocol: an initial denaturation step of 94°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, elongation at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were analyzed on 1.5% agarose gels and visualized after staining with ethidium bromide. The size of the amplified product was 400 bp.

Dose-effects of antibody

To evaluate the impact of the antibody, 1, 0.5, 0.25 or 0.125 μg of antibody was used to detect 10^4 MAP in PBS. All MAP detection procedures involved bacterial capture. Results were evaluated by PCR and quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Specificity of IMS-PCR

Specificity of antibody-conjugated MNPs and PCR was evaluated for *M*. *bovis* ATCC 35726; *M*. avium ATCC 35716; clinical isolation strains of *M*. *intracellulare*, *M*. *fortuitum*, *M*. *smegmatis*; B. subtilis KCTC 2023; and *B*. *sporothermodurans* KACC 10906. Mycobacterium species are frequently isolated from cattle in Korea and *Bacillus* species have the same Gram-staining properties as mycobacteria.

Figure 1. (A) *Image of scanning electron microscopy of magnetic nanoparticles*.

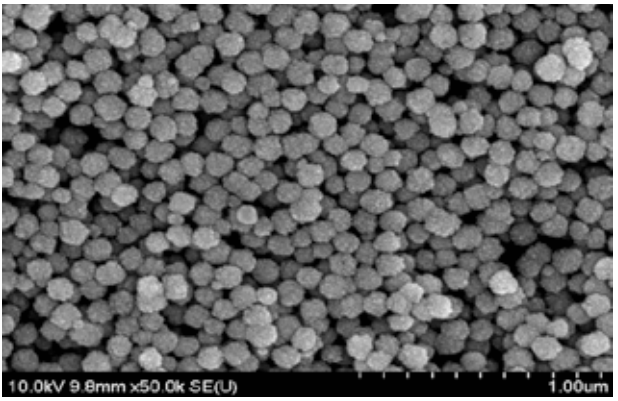
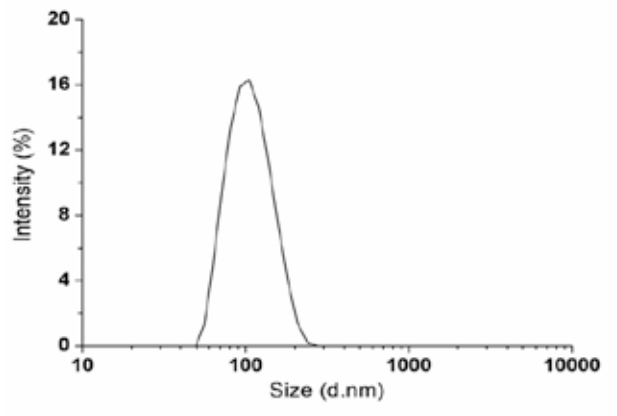


Figure 1. (B) *Particle size distribution of magnetic nanoparticles*.



Statistical analysis

Experiments were repeated three times and SPSS 12.0 was used for data analysis (SPSS, INC., Chicago, IL, USA). Statistical significance was determined by Student's t-test, with P-values < 0.05 considered significant.

RESULTS

Characteristics of MNPs and combination with MAP

An SEM image of MNPs (Fig. 1A) and the particle size distribution using DLS (Fig. 1B) revealed that the particles were uniform and monodisperse with a narrow size distribution. Size of MNPs was 90−110 nm in hydrodynamic conditions. The zeta-potential, which represents surface charge, was +32.1 mV and is shown in Table 1. SEM images of antibody-conjugated MNPs

Figure 2. *Scanning electron microscopic image of magnetic nanoparticles to capture M. paratuberculosis.*

are shown in Fig. 2. Several MNPs bound to a single bacterial cell and aggregated due to their magnetism. The amount of conjugated antibody was investigated using a bicinchoninic acid assay of pre-coupling and post-coupling solution. The results are summarized in Table 2. The binding ratio of anti-MAP chicken IgY with MNPs was 96.44 ± 0.77% (mean ± SD).

Bacterial detection and dose effect of antibody

The PCR results regarding limitation of bacterial detection were shown in Fig. 3. One MAP was detected using antibody-conjugated MNPs or PMBs combined with the IS900 PCR, even in the pooled stool environment. Upon converting into gram, the detection limit was 20 MAP cells g^{-1}. The MNPs and PMBs yielded same detection limit in four clinical isolation strains (data not shown).

The specificity of IMS-PCR using antibody-conjugated MNPs was evaluated with *M. bovis*, *M. intracellulare*, and *M. smegmatis*, as well as *B. subtilis* and *B. sporothermodurans*. False-positives due to antibody cross-reactivity and non-specific binding were primary concerns. Several non-targeted bacteria were chosen for analysis due to their genus or Gram properties. None of the chosen comparator samples showed reactivity with antibody-conjugated MNPs (data are not shown).

Also, detection efficiency at the different doses of antibody-conjugated MNPs or PMBs was investigated. 1, 0.5, 0.25 or 0.125 μg of antibody showed good efficiency to detect 10^4 MAP (Fig. 4A). At the same time, quantification of PCR bands was measured (Fig. 4B), and MNPs showed significantly effective values than PMBs using Student's t-test.

DISCUSSION

M. paratuberculosis infection of domestic

Figure 3. (A) *Results of immunomagnetic separation-polymerase chain reaction with IS900 primers. DNA of 10_5, 10_4, 10_3, 10_2, 10_1 or 1 M. paratuberculosis cells in pool stool solution (lane 1–6) or in PBS (lane 7–12) reacted with antibody-conjugated magnetic nanoparticles; P, positive control; N, negative control; M, 100 bp marker.*

Figure 3. (B) *Results of immunomagnetic separation-polymerase chain reaction with IS900 primers. DNA of 10_5, 10_4, 10_3, 10_2, 10_1 or 1 M. paratuberculosis cells in pool stool solution (lane 1–6) or in PBS (lane 7–12) reacted with antibody-conjugated paramagnetic beads; P, positive control; N, negative control; M, 100 bp marker.*

animals is associated with significant economic loss to the livestock industry. Strategies to prevent the spread of *M. paratuberculosis* in animal populations are hampered by the lack of rapid, accurate and sensitive diagnostic methods. Conventional fecal culture and serological assays are limited because they are effective only in clinical stages of infection.[4] Therefore, new approaches have been needed for fast and efficient detection of *M. paratuberculosis*.

Immunomagnetic separation is a rapid, specific, efficient, and simple method for directly isolating target organisms in clinical samples. Different reports have been published regarding the use of magnetic particles for IMS detection of *M. paratuberculosis*, but this is the first study to demonstrate the utility of nano-sized magnetic particles for this purpose. In previous studies, IMS has been used to detect 10_1 CFU of *M. paratuberculosis* in 50 mL cattle milk and 1 CFU in 50 mL cattle milk.[20, 21] In fecal samples, *M. paratuberculosis* detection limits are $2 \times 10_4$ cells g-1 and 10_1 CFU in 200 mg^{-1} feces.[18, 22] In comparison with conventional culture methods, IMS-PCR is a rapid and specific method for the detection of target bacteria in various matrices, even in the presence of PCR inhibitors.[23] The most widely used particle is the available paramagnetic Dynabeads®, which are micro-sized beads. However, nano-sized particles provide better capture efficiency because of their high surface-to-volume ratio and surface activity.[24, 25] Yang et al. used IMS with nanoparticles to detect *Listeria monocytogenes* in milk, and reported a capture efficiency 1.4 to 26 times higher than IMS with Dynabeads®.[26] Our results indicated that the MNPs conjugated with 0.125 to 1 µg antibody provided significantly higher detection efficiency.

The MNPs used in this study were uniform, monodisperse and silica-coated. Monodisperse nanoparticles with a narrow size variation provide better performance than polydisperse particles.[27] Silanization with amoinosilane on the surface of MNPs is formed by Fe−O−Si chemical bond and reported to improve the protein immobilization,[25] which means it can increase immunoglobulin immobilization. The immobilization is the most important step of a magnetic separation.[28] To minimize the aggregation of

Figure 4. (A) *Detection efficiency of different dose of antibody-conjugated paramagnetic beads or magnetic nanoparticles. DNA of 10_4 M. paratuberculosis detected with 1, 0.5, 0.25 or 0.125 µg of antibody-conjugated paramagnetic beads (lane 1–4) or magnetic nanoparticles (lane 5–8), respectively. P, positive control; N, negative control; M, 100 bp marker.*

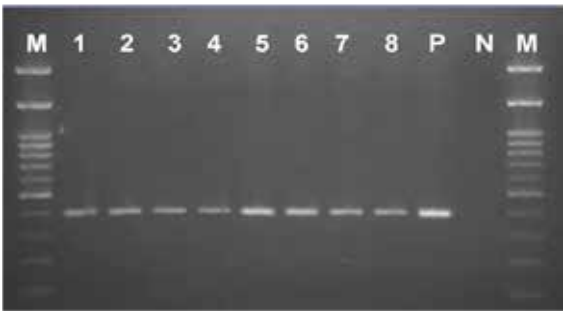
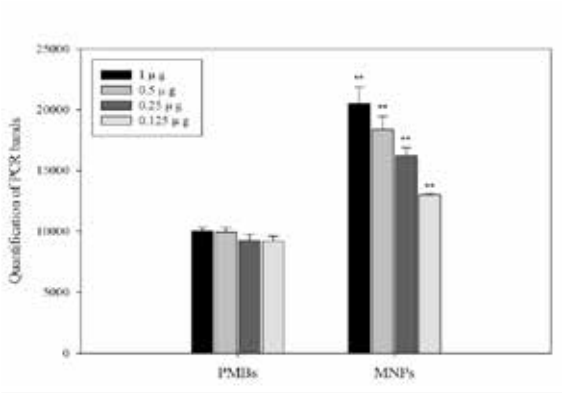


Figure 4. (B) *Detection efficiency of different dose of antibody-conjugated paramagnetic beads or magnetic nanoparticles. Quantification of band intensity. Graph is expressed as mean ± standard error. Comparisons between paramagnetic beads and magnetic nanoparticles were made using a Student's t-test (**P < 0.01).*



MNPs is necessary, silica can make weaken the particle-particle magnetic bipolar interaction, and one preferred functional group is amino group.[29] Moreover, the amount of nanoparticles is a key to IMS efficacy.[24] The binding ratio of MNPs to antibody determines the immunological ability of the antibody to detect the target bacteria. The binding ratio of antibody to MNPs was 96.44 ± 0.77% indicating efficient conjugation between bacteria and antibody.

Previously, false-negative results due to PCR inhibitors such as bile salts, bilirubin, and complex polysaccharides were a major limitation preventing accurate detection of bacteria in fecal samples;[22] difficulties in detecting bacteria at low concentrations and cross-reaction with non-target bacteria are limitations of IMS.[30] In this study, twenty of *M. paratuberculosis* ATCC 19698 cells g-1 were detected by IMS, even in the fecal environment. In addition, two clinical isolation strains from feces and two from intestine yielded the same detection limit as the standard strain. Thus, antibody reactions with specific bacteria eliminate the false-negative problem, enabling *M. paratuberculosis* detection with antibody-conjugated MNPs in the early stages of Johne's disease. Although anti-MAP chicken IgY cross-reacted with bacteria of the *Mycobacterium avium* complex as shown in a previous study,[10] nonspecific binding was eliminated with species-specific PCR primers. Moreover, MNPs are more cost-effective than paramagnetic beads. Cost-effectiveness reflects the improved treatment and cost of diagnostic method,[31] and the cost of magnetic beads was only considered due to same efficiency of MNPs and paramagnetic beads. Paramagnetic beads are four-fold more costly than MNPs. In addition, polyclonal IgY is relatively inexpensive and easy to produce in large quantities.[10]

In conclusion, new molecular tools using antibody-conjugated MNPs provide a rapid and cost-effective method for *M. paratuberculosis* infection in clinical samples; use of PCR reduces confirmation times from 12−16 week to 2−3 days.

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CONFLICT OF INTEREST

The authors declare that they have conflict of interest.

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