



Increased sensitivity of enterotoxigenic *Escherichia coli* detection in stool samples using oligonucleotide immobilized-magnetic nanoparticles

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ABSTRACT

PCR detection of enterotoxigenic *Escherichia coli* (ETEC) can be used directly on stool sample. However, it still has limitations due to presence of PCR inhibitors and interferences. This study, oligonucleotide primer specific to ETEC was immobilized onto MNPs and applied for separation and enrichment of ETEC-DNA from contaminants in stool after boiling. DNA separation efficiency was evaluated using conventional PCR and magneto-PCR-enzyme linked-gene-assay (MELGA). Due to high specificity of primer and efficiency of nanoparticles to bring down PCR inhibitors, DNA separation using primer-immobilized-MNPs exhibited 100-fold increase of sensitivity compared to that using simple boiling. Moreover, the sensitivities in stool were increased from 10^8 to 10^6 CFU/mL and 10^4 to 10^2 CFU/mL when PCR products were detected by gel electrophoresis and MELGA, respectively. Results suggested that oligonucleotide-immobilized-MNPs combined with boiling DNA extraction method was successfully used to separate the DNA of ETEC in stool with high sensitivity using MELGA.

1. Introduction

Diarrhea remains a cause of morbidity and mortality around the world and among all age groups [1]. It also continues to be a prevalent disease among travelers visiting developing countries. Diarrhea causes approximately 2.2 million deaths a year, most of which are in children under age five living in those countries [2, 3, 4]. When inadequate sanitization and poor hygiene of food and water cannot be avoided, infections occur easily. Acute diarrhea is usually caused by infection with enterotoxigenic *E. coli* (ETEC), rotavirus, *Vibrio cholerae* (*V. cholerae*), *Campylobacter*, or *Shigella* spp. [2, 3, 4].

In clinical diagnosis of diarrheal disease, several pathogens can be detected by conventional methods, but ETEC cannot be distinguished from other *E. coli*. Since ETEC is recognized as an *E. coli* [5,6] strain producing heat-labile and/or heat-stable (ST) enterotoxin(s) [7], detection of these enterotoxins has been used as a marker for ETEC identification (based on phenotypic assays such as ELISA, dot blot, slide agglutination and disk diffusion) [8,9]. However, these techniques are

labor-intensive, time-consuming and difficult to interpret. Genotypic assays include polymerase chain reaction (PCR) and DNA/DNA hybridization, and recently a combined genotypic-phenotypic assay (PCR-ELISA) has been developed [10,11]. Among these techniques, PCR was found to be useful for diagnosis of ETEC directly on stool as well as in isolated colonies [12]. Sensitivities of 10^3 to 10^7 colony-forming units (CFU)/mL have been reported for ETEC detection, varying from conventional multiplex PCR to real-time quantitative PCR [13, 14, 15]. PCR detection directly on stool is the best approach when rapid detection is required. However, the sensitivity of detection in such clinical samples is limited due to the presence of PCR and intrinsic inhibitors extracted along with pathogen DNA (such as complex polysaccharides, bile salts, lipids and urate) [16]. Therefore, separation and enrichment of target DNA from patient samples before attempting identification would be an effective way to increase the sensitivity of the detection.

In recent years, the excellence of magnetic nanoparticles (MNPs) in pathogen detection has been reported [17, 18, 19]. Due to their super-paramagnetic property, high surface-to-volume ratio, and surface

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presence of various functional groups, MNPs have been successfully applied to simplify handling processes and improve sensitivity of detection [20, 21, 22, 23, 24, 25, 26]. MNPs can be used as an efficient separation and enrichment tool, e.g. MNPs immobilized with oligonucleotides have been used for DNA separation during the detection process of pathogenic bacteria, viruses, as well as cancer cells [27, 28, 29, 30, 31]. These detection systems are of high sensitivity and specificity.

In this study, we aimed to develop a simple and convenient DNA extraction method to improve specificity and sensitivity of ETEC detection from stool samples. A forward primer which corresponded to the heat-labile toxin of LT gene of ETEC (LT-F) was immobilized onto MNPs using carbodiimide method. The primer immobilized MNPs (LT-F-MNPs) were then combined with a boiled DNA extraction method to enrich and separate target DNA from stool samples potentially containing PCR inhibitors. The efficiency of DNA separation in a buffer system and in stool samples by simple boiling combined with LT-F-MNPs was demonstrated using a conventional PCR. The sensitivity of ETEC detection using the enriched DNA from the functionalized MNPs was then evaluated and compared with the simple boiling DNA extraction method (without MNPs) by way of conventional PCR and a magnetopCR-enzyme linked gene assay (MELGA) [31]. The results showed that combining MELGA with the specific primer-bound MNPs enhanced the sensitivity of detection of ETEC in stool samples. This highly sensitive method provides rapid detection, which could be useful for identification, treatment and control of ETEC diarrhea.

2. Materials and methods

2.1. Bacterial preparation

Bacteria strains used in this study, including ETEC, *E. coli* (ATCC 25922), *V. cholerae* El Tor, *S. flexneri*, and *S. aureus* (ATCC 25923), were kindly provided by the Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology and Faculty of Pharmacy, Mahidol University, Thailand. Bacteria were grown on tryptic soy agar (TSA) (Merck KGaA, Germany) at 37 °C for 16–18 h. Three isolated colonies were subsequently collected for culture in tryptic soy broth (TSB) (Becton, Dickinson and Company, France) at 37 °C for 16–18 h with continuous shaking at 180 rpm. The bacteria were harvested by centrifugation 16,000 g and suspended in PBS (137 mM NaCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, pH 7.4, 2 mL). The suspended ETEC bacteria (OD_{600nm} of 1.0) were serially diluted for DNA extraction. The number of ETEC bacteria was determined by plating 100 µL of each dilution on TSA. The plates were incubated at 37 °C for 16–18 h. Finally, colonies were counted to determine colony forming units per milliliter (CFU/mL).

2.2. Preparation of LT-F immobilized onto MNPs

LT-specific primer, conjugated with amino group at 5' end (NH₂-LT-F: 5'-NH₂-C₆-GGC GAC AGA TTA TAC CGT -3') (Bio Basic Canada Inc., Canada), was immobilized onto the surface of carboxylated MNPs composed of Fe₃O₄ core in a polysaccharide matrix (hydrodynamic size 245.3 ± 2.6 nm) (FluidMAGARA, Chemicell, Germany) using the carbodiimide method [31]. Morphology of the carboxylated MNPs investigated using Transmission electron microscope (TEM, JOEL, JEM-2010) is shown in Fig. 1. Particle size and size distribution in dried state (*n* = 100) were 9.0 ± 1.9 and 1.14, respectively. Number of microequivalents of acid groups per gram and surface carboxyl groups originated from glucuronic acid determined using conductometric back titration were 1032.0 ± 56.6 µeq/g and 431.5 ± 23.3 µC/cm², respectively [31,32].

Before immobilization, the carboxylated MNPs (1 mg) were washed twice with 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (25 mM, pH 6.0, 500 µL). Next, amino modified LT-F (5 nmol) dissolved in MES buffer was added to the MNPs. The mixture was incubated at room temperature for 30 min with gentle shaking. After incubation, freshly

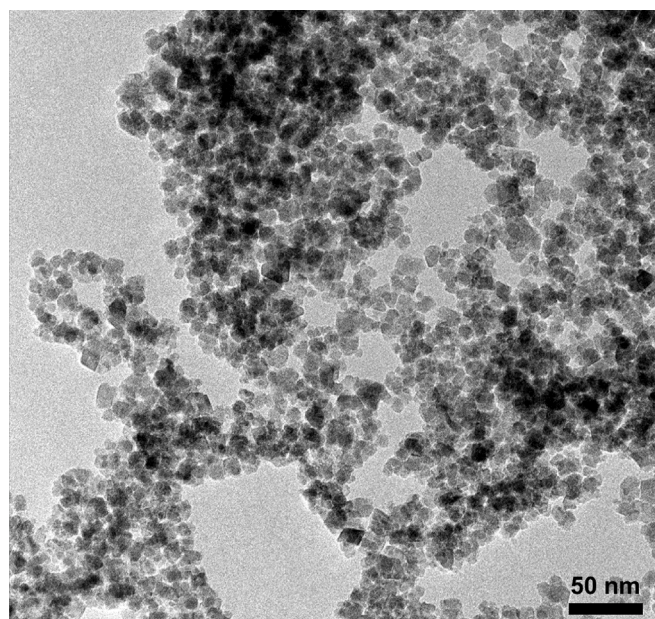


Fig. 1. TEM micrograph of the carboxylated MNPs.

prepared *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (protein sequence grade, Sigma-Aldrich, U.S.A.) (10 mg/mL, 10 µL) was added to activate the carboxylic acid groups of the MNPs. MES buffer was subsequently added to bring the final volume to 100 µL before incubation at 4 °C for overnight. Then, LT-F-immobilized MNPs (LT-F-MNPs) were separated by applying magnetic force, and supernatant was collected to measure the residual primer concentration using the Nanodrop (Thermo Fisher Scientific Inc., U.S.A.). The amount of immobilized NH₂-LT-F on the MNPs (nmol of primer/mg of MNPs) and binding efficiency were determined as previous studies [28,33]. The LT-F-MNPs were incubated with Tris (tris hydroxymethyl aminomethane) buffer (50 mM, pH 7.4, 500 µL) at RT for 15 min in order to quench the unreacted activated carboxyl groups. After the supernatant was discarded, the LT-F-MNPs were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA containing 0.1% BSA, pH 8.0, 50 µL) [34] to obtain final concentrations of 20 mg/mL LT-F-MNPs and stored at 4 °C.

2.3. Bacterial DNA extraction

Bacterial DNA was extracted by a conventional phenol-chloroform method following the manufacturer's recommendation (TRIzol, Invitrogen, USA). DNA concentration was determined by measuring absorbance at 260 nm using a Nanodrop 2000 (Thermo Fisher Scientific). To determine the efficiency of DNA extraction using MNPs, the LT-F-MNPs were used in a boiling DNA extraction method. ETEC suspended in binding and washing (B&W) buffer (5 mM Tris, 0.5 mM EDTA, 1 M NaCl, pH 7.5, 1 mL) was boiled at 100 °C for 5 min and immediately chilled on ice for 5 min. Then, the LT-F-MNPs (2 mg/mL, 25 µL) were added to the boiled bacterial suspension and left for DNA hybridization at 50 °C for 2 h. After that, DNA-bound LT-F-MNPs were magnetically collected and washed twice with B&W buffer (500 µL) prior to dissolving with TE buffer (10 µL). The DNA-bound LT-F-MNPs were stored at 4 °C until used.

In the case of direct DNA extraction from stool samples, an experiment was carried out based on a heat-shock method using a fresh stool sample collected from a healthy child donor and immediately stored at 20 °C [35]. Thawed stool sample (4.0 g) was homogenized aseptically for 5 min. A 10 mg sample of the mixture was randomly picked up, spiked with ETEC suspended in B&W buffer (1 mL), and vigorously homogenized for 30 s prior to DNA extraction with a procedure similar to that used in the case of bacteria suspended in buffer. The overall

process of DNA extraction using LT-F-MNPs is illustrated in Fig. 2.

In parallel, the simple boiling method was carried out by incubating a bacterial suspension at 100 °C for 5 min and then chilling it on ice for 5 min. The suspension was centrifuged at 19,000 g at 4 °C for 20 min to collect supernatant containing genomic DNA. Good quality of genomic DNA, i.e., the ratio of absorbance at 260 to 280 nm ranged from 1.6 to 1.8, was stored at 4 °C for further use.

2.4. Conventional PCR and PCR inhibition assay

A pair of primers used to amplify a 440 bp segment of LT gene (Bio Basic Canada Inc., Canada) was synthesized as previously reported [36, 37]. To confirm specificity of the primer to ETEC, PCR was performed against various bacteria including ETEC, *E. coli*, *V. cholerae* El Tor, *S. flexneri*, and *S. aureus*. A mixture (50 µL) containing PCR buffer [1 × 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1 mg/mL BSA, 10 mM (NH₄)₂SO₄ and 1.5 mM MgCl₂], dNTP (0.1 mM), LT forward primer (LT-F: 5'-GGC GAC AGA TTA TAC CGT-3', 0.1 µM), LT reverse primer (LT-R: 5'-CGG TCT CTA TAT TCC CTG-3', 0.1 µM), Taq DNA polymerase (1.25 units, RBC Bioscience, Taiwan), and DNA template or DNA-MNPs complex, was employed for DNA amplification using a thermal cycler (Bio-Rad Laboratories, USA). Nuclease-free water was used as negative control instead of DNA template. Optimal PCR conditions were carried out as follows: preheat at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 50 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. The PCR products were analyzed using 1.2% agarose gel electrophoresis.

To observe whether the carboxylated MNPs used in this study interfere amplification reaction, PCR inhibition assay was carried out using various concentrations of the particles (10, 20, 30, 40, 50, and 60 µg) to amplify LT gene followed the same procedure as conventional

PCR. PCR products were then visualized by agarose gel electrophoresis.

2.5. MELGA

MELGA technique was performed following the protocol described in the previous study [27,31,38] (Fig. 2). Briefly, the technique employed LT-F-MNPs and biotinylated-reverse primers (5'-biotin-CGG TCT CTA TAT TCC CTG-3') in the DNA amplification. Reaction conditions of the amplification were the same as the conventional PCR conditions described above. After DNA amplification, the amplified products (biotin-amplicon-MNPs) were conveniently separated from contaminants using a permanent magnet and mixed with streptavidin-horseradish peroxidase (SA-HRP) (0.1 µg/mL of SA-HRP, 50 µL) (KPL, Kirkegaard & Perry Laboratories (KPL), Inc., USA). The mixture was then incubated at RT for 1 h, in a dark cabinet, allowing complete biotin-SA interaction. After that, the HRP-SA-biotin-amplicon-MNPs products were collected using a permanent magnet and washed twice with DEPC-treated water. Then, 2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) (50 µL) (ABTS, KPL, Inc., U.S.A.) solution containing H₂O₂ (50%) was added and incubated at RT for 1 h in a dark cabinet to allow an HRP-catalyzed oxidation reaction. Finally, absorbance of oxidized ABTS was measured at 405 nm using a Nanodrop 2000 (Thermo Fisher Scientific) and compared with negative control (DNA-free sample). The results of MELGA detection were presented as relative absorbance change.

3. Results

3.1. Specificity of ETEC primer

The specificity of the ETEC primer was tested using various bacteria

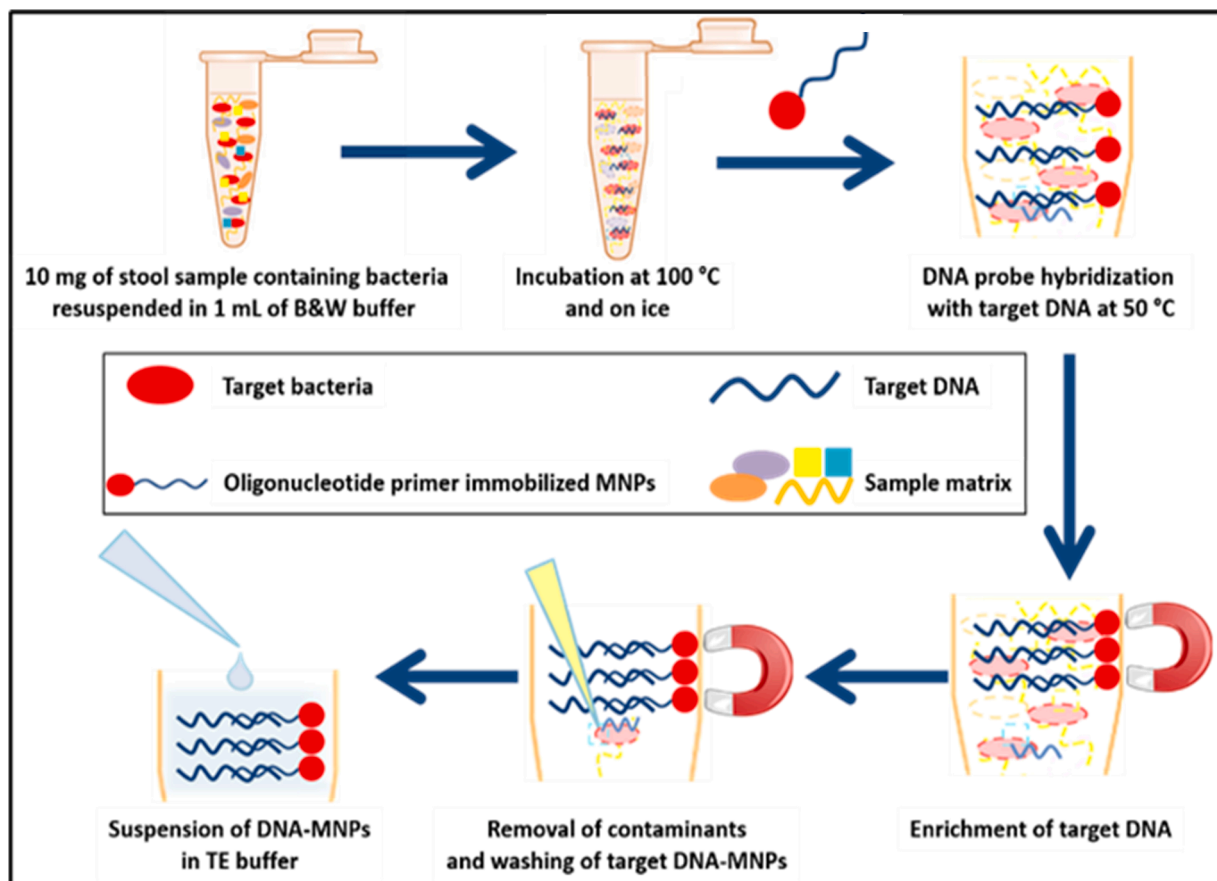


Fig. 2. Schematic representation of DNA extraction and enrichment using oligonucleotide primer immobilized MNPs (LT-F-MNPs).

including ETEC, *E. coli*, *V. cholerae* El Tor, *S. flexneri*, and *S. aureus*. Genomic DNAs were extracted by phenol-chloroform technique (TRIzol reagent) and adjusted to 1 µg/µL of DNA prior to PCR. PCR products were separated on agarose gel electrophoresis showing a specific band of the LT segment amplified from only ETEC genomic DNA (Fig. 3). This indicated that the selected pair of primers was specific to only the DNA of ETEC when compared with DNA of non-toxicogenic *E. coli* and other food-borne pathogens including *V. cholerae* El Tor, *S. flexneri* and *S. aureus* as reported previously [36,37].

3.2. Immobilization of forward primer onto MNPs

The specific forward primer conjugated with amino group (NH₂-LT-F) was covalently bound to the carboxyl-modified MNPs by way of the carbodiimide method [27,28,31]. After coupling reaction, the average amount of LT-F immobilized onto MNPs (LT-F-MNPs) calculated according to the previous study was 4.92 nmol/mg (mean±SD = 4.92±0.01) and the binding efficiency was 98.32% (mean±SD = 98.32±0.04) [31,33]. This result indicates that most LT-F were immobilized on the surface of MNPs. LT-F-MNPs could be used to extract target DNA and subsequently detect it by the MELGA method.

3.3. PCR inhibition assay

Various concentrations of carboxylated MNPs was added in PCR amplification of LT gene ranged from 10 to 60 µg. The result is presented in Fig. 4. It can be clearly seen the band specific of LT gene of ETEC in all concentrations (10–60 µg of added carboxylated MNPs) compared to negative control (No MNPs), this suggests no inhibition effect from the carboxylated MNPs to PCR process.

3.4. DNA extraction and enrichment using LT-F-MNPs

To evaluate performance of the hybridization probe (LT-F), DNA extraction using LT-F-MNPs was performed on stool and on buffer solution spiked with ETEC (10⁸ CFU/mL). LT-F-MNPs were added to stool and buffer samples containing target DNA released from bacterial cells after heat-shock. The mixture was incubated allowing hybridization of complementary DNA, and the DNA-MNPs complexes were then collected by applying an external magnetic field. The occurrence of

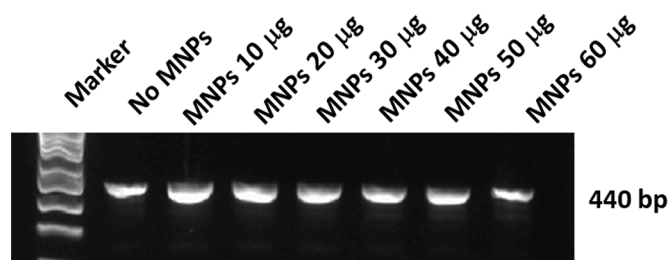


Fig. 4. PCR inhibition assay using various concentrations of the carboxylated MNPs.

DNA-MNPs complexes was subsequently monitored by conventional PCR-gel electrophoresis and compared with the extracted DNA obtained from the simple boiling method as shown in Fig. 5. The result showed band specific of LT-F to ETEC from both ETEC spiked stool and in buffer solution sample at 440 bp compared to positive control using phenol chloroform.

3.5. Sensitivity of ETEC detection in stool by MELGA using DNA extraction by LT-F-MNPs

Since the most severe diarrhea cases caused by ETEC infection are in children [1], in this study baby's stool was used as a sample. Aliquots of 10 mg of the stool sample (found to be an optimal sample size giving a high sensitivity of bacterial DNA detection as well as presenting a low amount of contaminants which could interfere with detection [35]) were spiked with various ETEC cell concentrations (10⁰–10⁸ CFU/mL). Sensitivity of the DNA detection from boiling DNA combined with LT-F-MNPs was determined and compared with that using a simple boiling method without MNPs [by way of conventional PCR or MELGA as shown in Fig. 6A-D]. The result showed the lowest amount of DNA used to amplify and reveal by gel electrophoresis and MELGA using simple boiling were 10⁸ and 10⁴ CFU/mL, respectively (Fig. 6A, B) whereas the boiling method in combination with MNPs were 10⁶ and 10² CFU/mL, respectively (Fig. 6C, D).

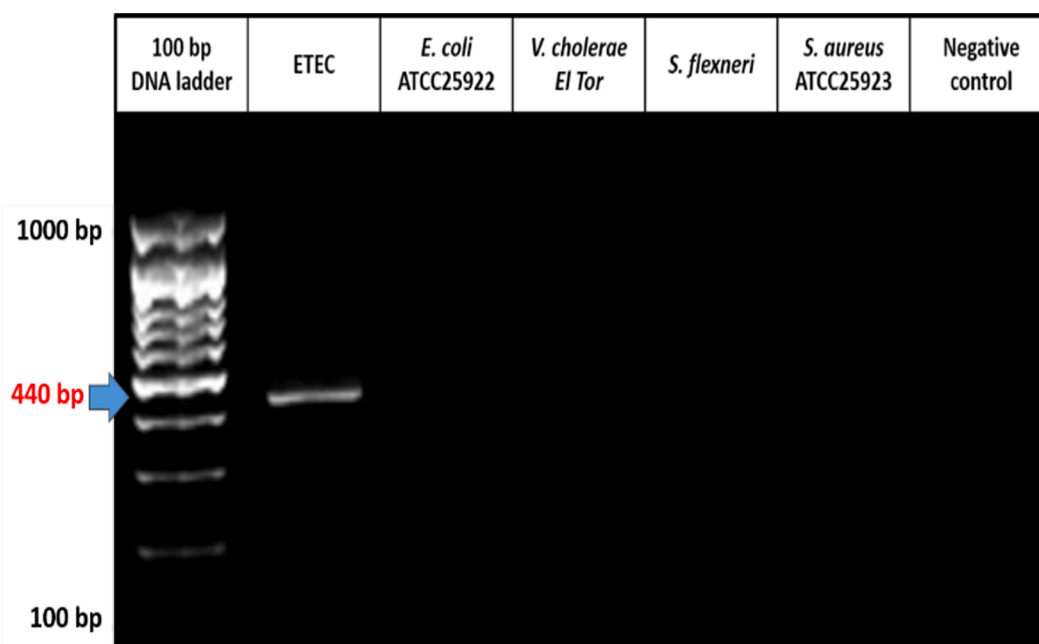


Fig. 3. Specificity of the LT primer using conventional PCR against various bacterial DNAs extracted by TRIzol reagent followed by agarose gel electrophoresis.

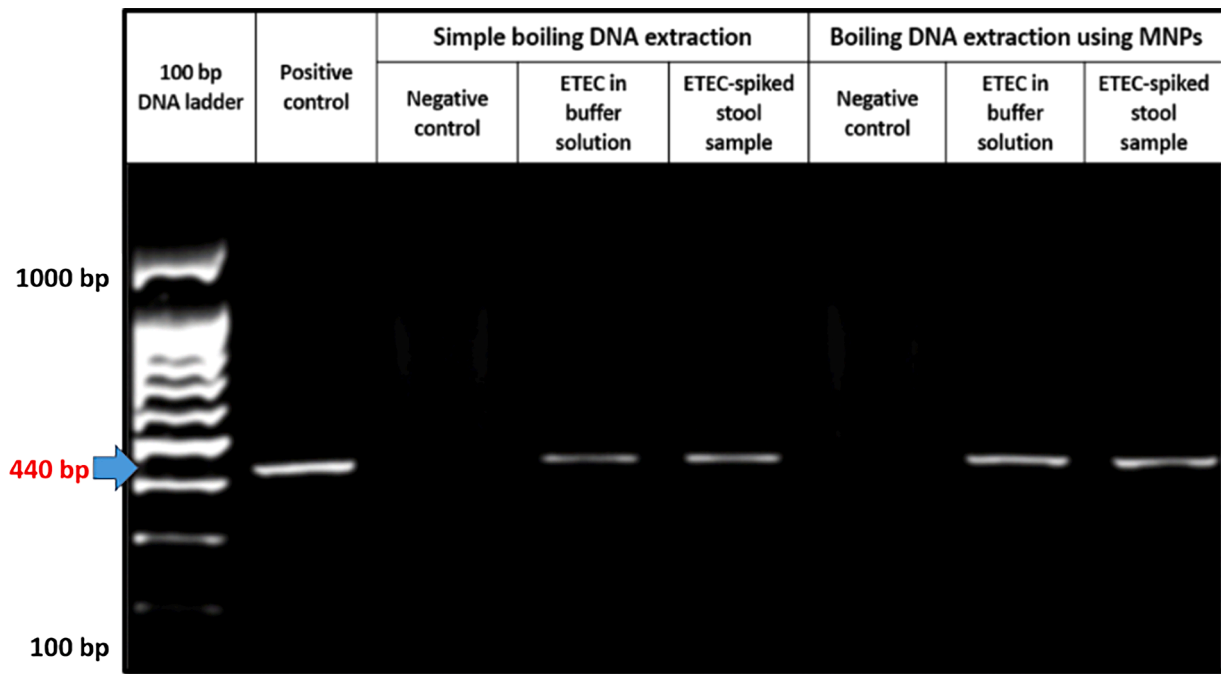


Fig. 5. Agarose gel electrophoresis of DNA extraction using the simple boiling method with and without LT-F-MNPs at the same ETEC concentration (10^8 CFU/mL) spiked into buffer solution and stool samples. ETEC DNA (500 ng) extracted by TRIzol reagent was used as positive control.

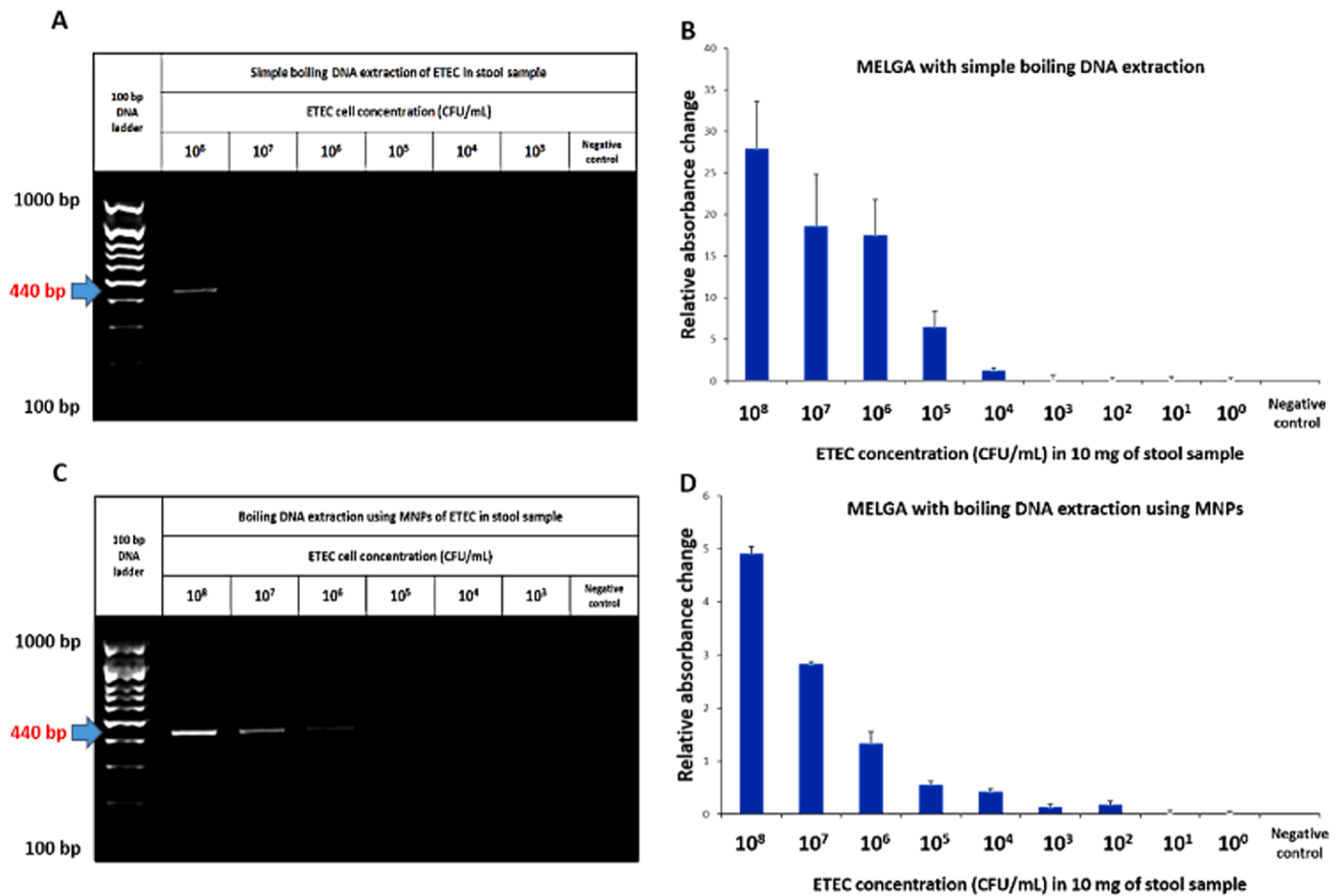


Fig. 6. Sensitivity of ETEC detection using conventional PCR (A and C) and MELGA (B and D) from simple boiling DNA extraction with (C and D) and without (A and B) LT-F-MNPs.

4. Discussion

The molecular size of the amplified LT gene segment of ETEC was 440 bp and belonged to a part of the heat-labile enterotoxin A subunit (corresponding to position 153 to 592 of GenBank ID: DQ778054.1) which is located between two highly conserved regions, 236- and 280-bp [39]. ETEC infection causes 'cholera-like diarrhea'. As reflected in its name, ETEC produces a cholera toxin (CT)-like enterotoxin which was identified later as LT [40]. Although the LT of ETEC has multi-similarities (i.e. physiology, structure, antigenicity and mode of action) to those of CT produced by *V. cholera* [41], our result showed that the CT-producing *V. cholerae* was not detected using this LT primer pair. This result suggests that the primer pair was highly specific to LT-producing ETEC strains. Therefore, it is suitable for using as an oligonucleotide probe for the binding and separating of ETEC DNA. Then, this forward primer was chosen for immobilization onto the surface of MNPs and for use in the next steps.

Results from simple boiling DNA extraction and from boiling DNA extraction combined with LT-F-MNPs show that the band of PCR products was obtained from both ETEC-spiked stool and buffer solution samples at the expected size (440 bp) compared to a positive control using phenol-chloroform extracted DNA (500 ng). This indicated not only the efficiency of DNA extraction by the boiling technique but also the strong binding of primer to specific target DNA driving the PCR reaction. It is worth noting that the DNA enriched by LT-F-MNPs which were complementarily bound to the oligonucleotide probe and caught on the MNP surface can be further amplified using conventional PCR. Results from PCR inhibition assay indicated that the amount of MNPs used in this experiment did not interfere with PCR reaction [42]. The band intensity fraction was further quantitated by GelQuant. NET software v.1.7.8 provided by biochemlababsolutions.com. It was found that the band intensities in the case of boiling DNA extraction combined with LT-F-MNPs were higher than those obtained from simple boiling technique (1.85-fold in buffer samples and 1.29-fold in stool samples). This was because the LT-F-MNPs separated the target DNA from inhibitory contaminants and enriched target DNA on the matrix. As a result, template DNA was of higher quality and favored the PCR reaction leading to more amplified product. Thus, we suggest that the LT-F-MNPs can be used to directly extract the DNA of ETEC from stool samples and reduce interferences during the target amplification [43, 44, 45, 46].

After simple boiling DNA extraction, the sensitivities of PCR and MELGA detection was 10^8 and 10^4 CFU/mL, respectively (Fig. 4A-B). Interestingly, using LT-F-MNPs in combination with the boiling method, the sensitivity of PCR and MELGA detection were increased to 10^6 and 10^2 CFU/mL, respectively (Fig. 4C-D). These results revealed that combining the LT-F-MNPs in boiling DNA extraction can yield sensitivities 100-fold higher than those using the simple boiling DNA extraction method alone. This indicated that the LT-F-MNPs specifically separated and enriched the target DNA, and thus resulted in a higher sensitivity of the quantitative detection using MELGA. This detection method was more sensitive than the quantitative PCR currently used for detection of ETEC (10^3 CFU/mL of bacteria) [15] as well as more sensitive than other developed detection methods [30, 47, 48]. Recent study utilized bacteriophage-conjugated magnetic beads to capture and separate *E. coli* BL21 from water samples, then quantified by using colorimetric method. They reported that 10^4 CFU/mL of *E. coli* was detected in 2.5 h which was comparable to our method. Moreover, they also incorporated their method with a pre-enrichment step to increase sensitivity to 10 CFU/mL, however this step also increased much more detection time (~ 8.5 h) compared to our method (~ 5.5 h) [49]. Our system was not only used to separate the target DNA from PCR inhibitors and interference matrix, but also to enrich the target DNA prior to PCR amplification. In addition, the detection limit of this system (10^6 CFU/g of stool by PCR-agarose gel and 10^4 CFU/g of stool by MELGA) covers the range of ETEC commonly present in both acute ETEC diarrhea patients (10^7 – 10^9 CFU/g of stool) and asymptomatic persons (10^4 – 10^5

CFU/g of stool) [15].

5. Conclusion

A simple and convenient DNA extraction method based on primer immobilized MNPs (LT-F-MNPs) was successfully developed and used to directly extract DNA of ETEC from stool samples. The LT-F-MNPs allowed specific separation and enrichment of DNA, and improved sensitivity of DNA detection. Sensitivity of MELGA detection with the boiling DNA extraction using LT-F-MNPs was higher than those of conventional PCR. This highly sensitive method should be evaluated further with clinical samples before deploying in clinical laboratories. Using MNPs in DNA extraction, the detection of ETEC can be applied in other complicated samples such as food, water, and soil. The detection method can be applied in laboratories having PCR machines to identify the bacteria strain. Therefore, the method could be appropriate for early detection and epidemiological survey of ETEC elsewhere. The sensitive detection of ETEC will be useful for improved prevention and control of ETEC diarrhea. Moreover, this method can be adapted easily for detection of other bacterial pathogens by modification of the specific primer.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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