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ORIGINAL ARTICLE

Design of ELISA-based diagnostic system for detection of enterohaemorrhagic *Escherichia coli*

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ABSTRACT

Background and Objectives: *Escherichia coli (E. coli)* O157:H7 is an intestinal pathogen of humans and animals, which causes serious gastrointestinal, urinary tract infection and hemolytic uremic syndrome. Connecting to the host cell is important in pathogenesis. EspA, Intimin and Tir proteins (EIT) are the most important bacterial features in the process of binding. These antigens can be very useful in detecting these bacteria. The aim of this study was to produce recombinant EspA, Intimin and Tir proteins (rEIT) to detect pathogenic *E. coli* O157:H7 by means of ELISA method.

Materials and Methods: The *eit* recombinant gene was expressed using IPTG in *E. coli* BL21 (DE3) and evaluated by western blotting. The purified rEIT protein was injected to rabbits and mice subcutaneously. Purified antibody was evaluated using indirect, competitive and sandwich ELISA confirming the precise detection of *E. coli* O157: H7.

Results: Indirect, competitive and sandwich ELISA specifically detected *E. coli* O157:H7 and each methods had the ability to identify more than 10^4, 10^4, 10^3 bacteria. The specificity of this method was evaluated by Entroheamoragic *E. coli*, enterotoxygenic *E. coli*, *Klebsiella pneumoniae*, *Vibrio cholera* and *Acinetobacter*.

Conclusion: These methods are the fastest, most accurate and cost effective methods for diagnosis of *E. coli* O157: H7, comparing to the conventional methods.

Keywords: *Escherichia coli* O157:H7; Indirect enzyme-linked immunosorbent assay (ELISA); Sandwich ELISA; Competitive ELISA

INTRODUCTION

The World Health Organization has estimated that around 1.8 million individuals die every year from diarrhea, which is usually caused by foodborne contaminated foods or drinking water (1). One of the pathogens that causes diarrhea is enterohemorrhagic *Escherichia coli* (EHEC), the most common cause of severe foodborne diseases (2, 3).

Among the enterohaemorrhagic strains, serotype

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O157:H7 is the most common cause of hemolytic-uremic syndrome (HUS) (4). The most important factor in the pathogenicity of EHEC is its attachment to the intestinal epithelial cell through EspA, intimin, and Tir proteins produced by the genes in the pathogenicity island of locus of enterocyte effacement (LEE). The EspA protein is a structural protein and a central component of a large filamentous organelle that interacts with the host cell, plays a role in the construction of the communication channel, and is essential for the translocation of Tir into host cells. Finally, the Intimin protein binds to the Tir and connects the bacteria to the host cell. This connection causes cell wall destruction and A/E lesions in the host (5). After binding the bacteria to intestinal epithelial cells and A/E lesions, the generated Shiga-like toxin enters the bloodstream and attaches to specific tissues, particularly the kidney, leading to uremic syndrome (6, 7). EHEC, also called as verotoxigenic *E. coli* Stx1, has many similar characteristics to the Shiga toxin produced by some *Shigella dysentery* type 1 strains. The two poisons are different in terms of their antigens and genetic makeup (8-10). EHEC strains are transmitted through contaminated water and food. Domestic ruminants, especially cattle and goats, are the main reservoirs of this bacterium (11). The most notable foods that can be infected with *E. coli* O157 in different epidemics are hamburgers, minced meat, milk, yogurt, cheese, vegetables, juices (especially apple juice), radish buds, and alfalfa (12). These animals' contaminated meat and dairy products are the primary source of human infection, especially in developed countries (13, 14). The accuracy and rapid detection of *E. coli* O157:H7 in humans and the public health and clinical management perspective are very important. In the early stages, rapid diagnosis of cases and prompt notification from health authorities are essential for effective epidemiological interventions. Polymerase chain reactions (PCRs), including simple PCR, multiplex PCR, and real-time PCR (15, 16), are commonly used for rapid detection of *E. coli* O157:H7 but require complex setups and well-trained personnel.

Enzyme-linked immunosorbent assay (ELISA) can quantitatively detect immunoglobulin G and was introduced in 1971 (17). Conventional ELISA has high reproducibility and potential for the simultaneous quantification of a significant number of assays and is widely used to detect the presence of particles, including bacteria (18), viruses (19), proteins (20), and pesticides (21). However, the ELISA detection limit for *E. coli* O157:H7 is 10^5 to 10^7 CFU mL^{-1} which is inadequate when the infectious dose is lower than 100 cells (22).

Although multiple sensitive methods for detecting *E. coli* O157:H7 have been reported, they usually require expensive equipment or skilled personnel and are time-consuming. EspA, intimin, and Tir proteins are specific surface antigens used for identification of *E. coli* O157:H7. As mentioned in a study by Amani et al., these three antigens are immunogenic and cause strong antibody production in mice. Therefore, in this study, we produced recombinant EspA, intimin and Tir proteins (rEIT) for detection of *E. coli* O157:H7 using three ELISA methods by purified antibodies which were produced in mouse and rabbit (An immunological study on the immunized rabbits and mice showed that humoral immunity in these animals was well stimulated) (23). Also, in this study EHEC, ETEC, *Klebsiella pneumoniae*, *Vibrio cholera*, and *Acidobacteria* (due to having the same antigens) were used to evaluate and determine the specificity of ELISA kit.

MATERIALS AND METHODS

Design and construction of *eit* chimeric gene. The synthetic gene encoding *eit* (EspA, intiman, Tir) for production of the EIT protein was designed with the 6XHis-tag at the N-terminal based on the previous study (23).

Expression of recombinant EIT protein. For expression, the synthetic *eit* gene was subcloned into pET28a and transformed into *E. coli* BL21 (DE3) (23). *E. coli* containing pET28-*eit* were grown at 37°C then, subcultured in LB broth containing kanamycin (40 µg/mL) and incubated for 2 h at 37°C and 150 rpm. After reaching the bacterium growth turbidity of OD_{600} : 0.7, IPTG was added at the final concentration of 1 mM for 6 h at 37°C at 150 rpm. Bacteria were harvest by centrifugation at 5000 rpm for 5 min and lysed with 8 M urea. The samples were analyzed by SDS-PAGE 12%.

Purification of recombinant EIT protein. Following analysis of expression, purification of the protein was performed using Ni-NTA column (QIAGENE, USA). For this purpose, 50 ml of *E. coli* BL21 (DE3)

bacteria containing pET- *eit* induced by IPTG was centrifuged for 10 min at 5000rpm. The bacterial cell pellet was suspended in 6 ml of lysis buffer (50mM NaH2PO4, pH 8.0, 300mM NaCl, 10mM imidazole, 0.2mg/ml lysozyme) for 30 min. After centrifugation at 14,000rpm for 20 min, the supernatant was collected and loaded to the Ni-NTA column. The column was washed with washing buffer (30 mM imidazole containing 5% glycerol). Finally, it was eluted with 2 ml extraction buffer (300 µm imidazole containing 5% glycerol). The samples were analyzed by 12% SDS-PAGE gel and concentration of purified protein was determined by Bradford assay and they were stored at -20°C.

Western blot. Western blotting was carried out to confirm the chimeric recombinant protein. The purified proteins from 12% SDS-PAGE were transferred on a membrane (PVDF) using transfer buffer (39mM glycine, 48mM Tris-base, 0.037% SDS, and 20% methanol). The PVDF membrane was blocked with a solution containing 5% skim milk at 4°C overnight and washed with PBS/T (PBS contain 0.05% Tween 20). The membrane was incubated in a 1/10000 dilution of antiHis-tag in the PBS/T, with gentle shaking for 1h at 37°C then washed with PBST and then incubated in 1/50000 dilution of HRP-conjugated goat anti-mouse IgG antibody (SIGMA), with gentle shaking for 1h at 37°C. After washing, detection was carried out using HRP staining solution (DAB). Reaction was stopped by rinsing the membrane twice with distilled water (23).

Animal immunization. In this study, BALB/c mice were prepared from Pasteur Institute of Iran. Mice were divided into control and test groups. In the test group, 10 µg recombinant EIT was injected to mice subcutaneously three times, on days 0, 14, and 28 with complete and incomplete adjuvant. The control group was injected with PBS. Also 120 µg of recombinant EIT protein with complete and incomplete Freund's adjuvant was injected into New Zealand rabbits in three steps. Blood sampling was performed two weeks after each injection. For serum isolation, the samples were centrifuged at 1500 rpm for 10 minutes.

Determination of antibody titer by indirect ELISA. Specific antigen-antibody responses were determined by ELISA method. The wells of polystyrene 8-well plates (MaxiSorp microtiter plates) with 3 µg of EIT antigen were covered by coating buffer (64 mM Na2 CO3, 136 mM NaHCO3, pH 9.8) and then were kept at 4°C overnight. Following washing and blocking with 100 µL/ well blocking solution (5% milk in TBST) for 1 hour at 37°C, 100 µl of HRP labeled anti-mouse and anti-rabbit anti-bodies diluted 1/2000 in PBST was added to each well. The plates were incubated at 37°C and washed three times with PBS/T. 100 µl of TMB substrate (Sigma) was added to each well stopped by 100 µL of 2 M sulfuric acid solution and the sample absorption was recorded at 450 nm wavelength by ELISA microplate reader (24). All ELISAs were conducted at least three times for confirmation.

Purification of IgG from mouse and rabbit serum using G column. Protein G column was used to purify IgG antibody (Sigma). At first, the column was washed with 100 mM Tris-HCl solution (pH=8). The column was then equilibrated with a 10 mM Tris-HCl (pH=8) buffer. Serum samples were loaded to the column and were washed with a 100 mM Tris-HCl (pH=8) buffer and then washed by a 10 mM Tris-HCl buffer (pH=8). The antibodies were eluted with 100 mM glycine (pH=3). The purified antibody was confirmed by 12% SDS- PAGE.

Evaluation of purified antibodies. After purification of mouse and rabbit antibodies, ELISA assay was carried out. According to the previous procedure, different concentrations of anti-EIT IgG Antibody (1000, 500, 250, 125, 62, 31, 15, and 7 ng) with constant concentration of antigen (3 µg) were used. In other case, an ELISA with different concentrations of antigen (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, and 1000 µg) and constant concentration of antibody were used.

Evaluation of various ELISA assay: sandwich ELISA method with *E. coli* O157: H7. Serial dilutions of 10^8 to 10^1 CFU/mL of *E. coli* O157: H7 were used for ELISA assay. Wells were coated with of 5 µg of mouse and rabbit antibodies with coating buffer, and then incubated at 4°C O/N and blocked with blocking buffer, then 200 ng to 303 ng of mouse and rabbit antibodies were added to each well. The plates were incubated for 1 h at 37°C. Following addition of the conjugated antibody with enzyme peroxidase (diluted 1/2000 in PBS/T), TMB substrate (Sigma)

was added to each well and incubated at 37°C for 15 min. The reaction was stopped by 2M sulfuric acid and absorption was recorded at 450 nm by ELISA microplate reader.

Indirect ELISA method with *E. coli* O157: H7. After bacteria were coated, 200 ng to 303 ng of mouse and rabbit antibodies were added to each well. The plates were incubated for 1 h at 37°C then conjugated anti-mouse and anti-rabbit antibodies were added to each well. The plates were read at 450 nm.

Competitive ELISA with specific antigen and bacteria. ELISA wells were coated with 3 µg of EIT antigens and 10^9 *E. coli* O157: H7 separately. Then Primary antibodies were individually added (1 to 2000 ng). After washing for 3 times, the non-bonding antibodies were removed. Then, a specific secondary antibody was added. TMB substrate (Sigma) was added to each well and the reaction was stopped by 2 M sulfuric acid and the sample absorption was read at 450 nm wavelength by ELISA microplate reader.

Specificity and sensitivity. The sensitivity was evaluated against five bacteria including *Klebsiella, Acinetobacter,* Enterotoxigenic *Escherichia coli,* Enterohemorrhagic *Escherichia coli,* and *Vibrio cholera* samples with dilution of 10^9 to 10^1 CFU /mL.

Clinical samples. 40 samples consisting of 30 urine and 10 stool specimens were collected from different health centers in Tehran. Indirect and sandwich ELISA test were performed with standard and clinical samples. These methods were done with EIT antigen (3µg) and 10^5 bacterial cells including of *E. coli* O157:H7 as positive control.

Statistical analysis. All data were obtained from three replicates and expressed as the mean and standard deviation (±SD) using SPSS 16 software. The data obtained from specificity test of ELISA were evaluated by SPSS and $p > 0.05$ was considered as significance.

RESULTS

Expression of recombinant EIT protein. Expression of the gene was performed with IPTG induction and the weight of the recombinant EIT protein was about 64.4 kDa on SDS-PAGE 12%. The results of optimizing the gene expression showed that the best expression was performed at 6 h, 37°C and 1 mM IPTG (Fig. 1A).

Purification of recombinant EIT protein. The recombinant EIT protein was purified using Ni-NTA column. As shown in Fig. 1B, molecular weight of the protein was 64.4 kDa.

Verification of recombinant protein by Western blotting. Western blotting was used to confirm the protein product. In this method, the His-tag antibody was used as shown in Fig. 1C.

ELISA for determination IgG titer. After collecting blood of immunized mice and rabbits, the results of ELISA showed a significant increase in antibody titer after each step of the injection compared to the control (Fig. 2).

Purification of IgG from mouse and rabbit serum using G column. Protein G column was used to purify IgG antibody. As shown in Fig. 3, the IgG antibody has molecular weight of 150 kDa, which is broken to two bands of 50 and 25 kDa.

Evaluation of purified antibodies titration using indirect and Sandwich ELISA method. Indirect and sandwich ELISA method was performed to determine the specificity of purified antibody. The cut off of mouse and rabbit antibody dilutions were 1000 and 2000 ng and *E. coli* O157:H7 bacterial cells were at 10^4 (Fig. 4).

Competitive ELISA with recombinant antigen, *E. coli* O157:H7 against antibodies. To evaluate the specificity of designed ELISA and determine its cut off, we made a competitive ELISA with recombinant antigen and bacteria. The result of competitive ELISA with antigen according to (Fig. 5A) showed that as the amount of recombinant antigen (rEIT) solution decreases with mouse antibody (1000 ng), a higher OD was produced, which means that a designed ELISA with up to 16 ng rEIT antigen can be identified.

Also, according to (Fig. 5B), the result of competitive ELISA with bacteria showed that, as the number of bacteria in incubated solution decreases with an over-mouse antibody concentration, the OD is increased, which means that the design of ELISA kit has

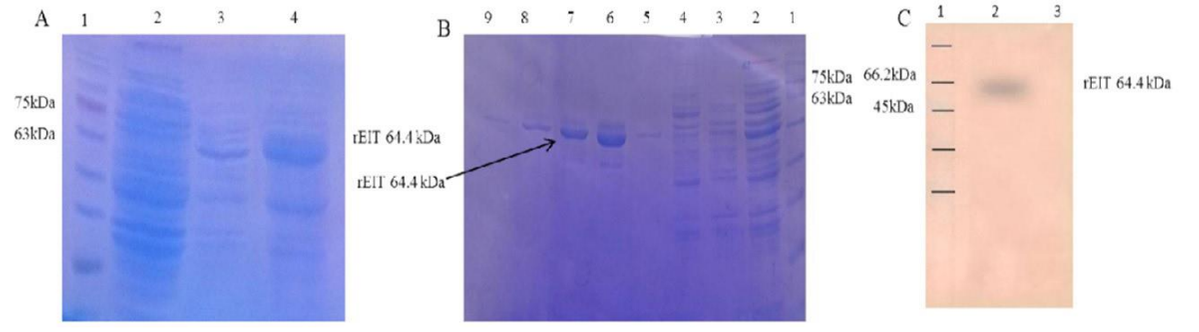

Fig. 1. Expression, purification and western blot analysis of recombinant EIT. A) Electrophoresis of the recombinant EIT protein: 1) Protein size marker, 2) Induced sample without IPTG, 3) Sample after induction (supernatant), 4) Sample after induction resulted from deposition (inclusion body). B) Purified recombinant protein with Ni-NTA column. 1) Protein size marker, 2) Extract from the lysate bacteria, 3) Flow through, 4) Washing buffer with 30 mM imidazole (pH:8), 5-8) Elution buffer with 300 mM imidazole and 5% glycerol, 9) MES buffer (20 mM). C) The confirmation of EIT recombinant protein by Western blotting product. 1) Protein size marker, 2) recombinant protein, 3) Non-induced as control.

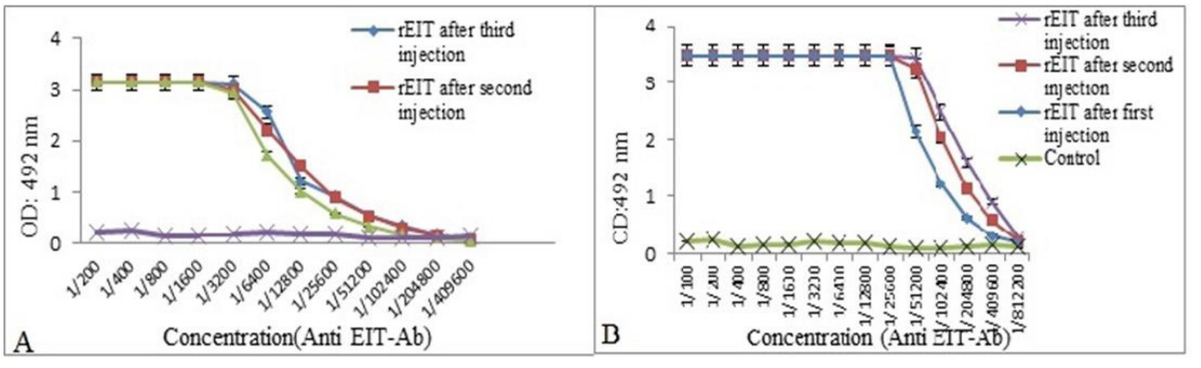

Fig. 2. Determination of antibody titer in mice and rabbits by ELISA method. A) ELISA for determination of antibody titer against recombinant protein in the mouse animal model. B) ELISA for determination of antibody titer against recombinant protein in the rabbit animal model.

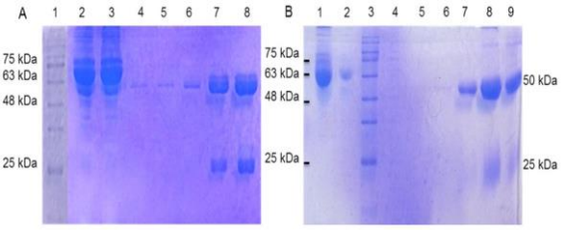

Fig. 3. Purification of antibody from mice and rabbits by G column. A) Purification mouse IgG antibody using G column, 1) Protein weight marker, 2) Flow through, 3) Washing buffer (pH=7.2), 4-8) Elution buffer with 100 mM glycine solution (pH=3). B) Purification rabbit IgG antibody using G column, 1) Flow through, 2) Washing buffer (pH=7.2), 3) Protein weight marker, 4-9) Elution buffer with 100 mM glycine solution (pH=3).

cut off= 10^3 bacteria and it does not detect less than this amount.

Evaluation cross-reaction of ELISA kit. To evaluate cross-reactivity, indirect ELISA was performed. *Klebsiella*, *Acinetobacter*, Enterotoxigenic *Escherichia coli*, Enterohemorrhagic *Escherichia coli*, and *Vibrio cholera* were used. The result was shown in Fig. 6.

Clinical samples. Indirect and sandwich ELISA were performed with standard and clinical samples which were collected from different health centers in Tehran. The samples included *E. coli* O157:H7 as positive and *Klebsiella* as negative control. The re-

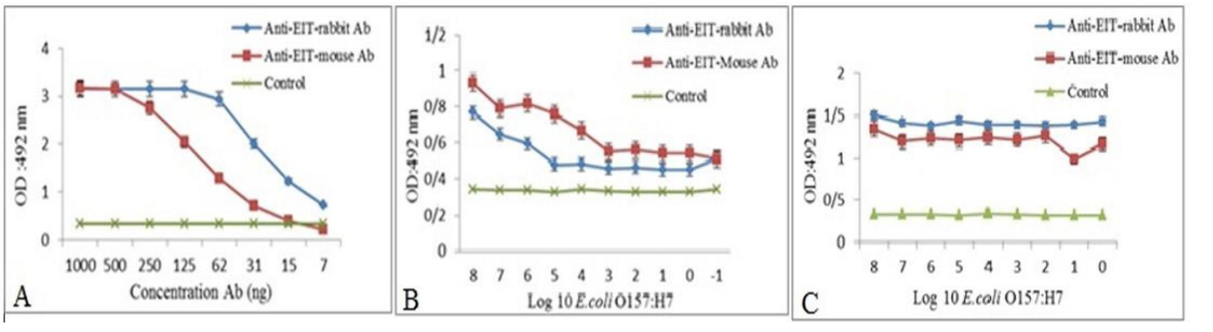

Fig. 4. ELISA for evaluating purified IgG. A) ELISA for determination of purified IgG titer produced in mouse and rabbit. B) Indirect ELISA using *E. coli* O157:H7 against with mouse and rabbit antibody. C) Sandwich ELISA using *E. coli* O157:H7 against mouse and rabbit antibody.

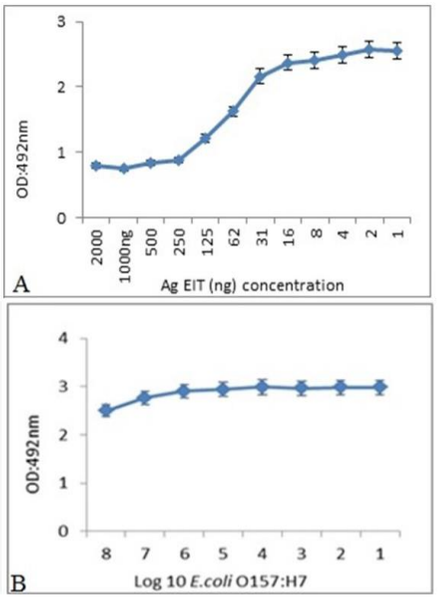

Fig. 5. Competitive ELISA with EIT antigen and *E. coli* O157:H7. A) ELISA using EIT antigen with mouse antibody. B) ELISA using *E. coli* O157:H7 bacteria with mouse antibody.

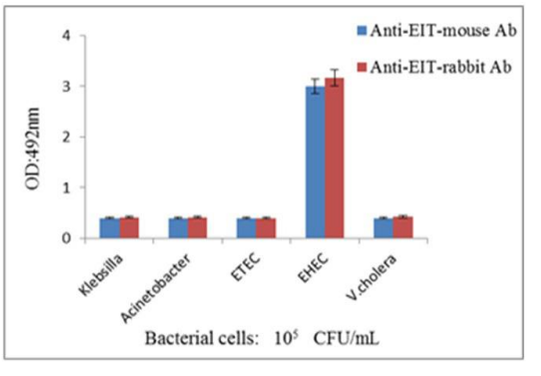

Fig. 6. Determination of specificity of ELISA using some bacteria with mouse and rabbit antibodies.

sults showed that *E. coli* O157:H7 can be detected by indirect and sandwich ELISA methods (Fig. 7).

DISCUSSION

Adhering to the host cell is an important step at the beginning of the pathogenic stage. Intimin, Tir, and EspA proteins are the most important surface antigens of *E. coli* O157:H7 in the binding process to host cells as well as A/E lesion formation (25). These three EIT recombinant proteins have high immunogenicity that can be very useful in detecting this invasive bacterium (23).

The *eit* recombinant gene was expressed in the *E. coli* BL21DE3 bacterium, and rEIT purified protein was injected into mice and rabbits subcutaneously. All three ELISA methods are specifically designed to detect *E. coli* O157:H7, and each designed kit with 16 ng of EIT antigen can detect more than 10^4, 10^3, and 10^3 bacteria, respectively.

In previous studies, Park et al. 1996 designed the ELISA method and compared it with the conventional culture method for detecting Enterohemorrhagic *E. coli* from stool specimens. They indicated that ELISA had a sensitivity and specificity of 91.2 and 99.5%, respectively, but the conventional method had a sensitivity and specificity of 97% and 95%, respectively (26). Also, Parma et al. 2012 designed a sandwich ELISA assay to detect Enterohemorrhagic *E. coli* (EHEC) based on a Shiga-like toxin with a detection limit of 115 ng/ml (27).

In the two studies mentioned (Park et al and Parma et al.), unlike our study, Shiga toxin was used as an antigen, and these methods can identify all toxin-producing strains. However, our study used sur-

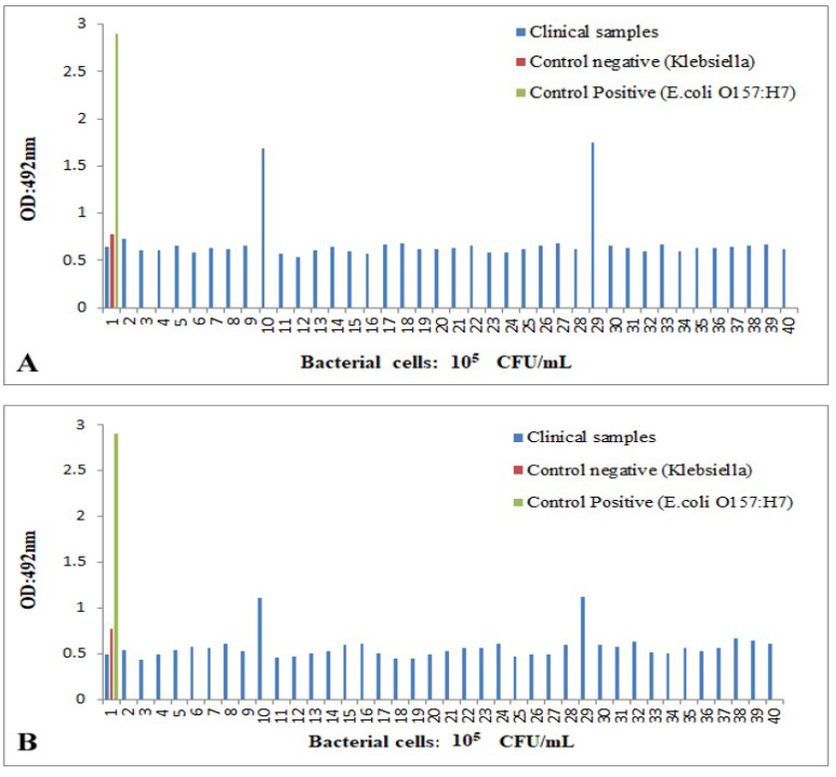

Fig. 7. Sandwich and indirect ELISA to evaluate clinical samples. A) Sandwich ELISA using clinical samples with rabbit antibody as capture antibody and mouse antibody used for detection. B) Indirect ELISA using clinical samples, positive and negative control with rabbit antibody.

face antigens of *E. coli* O157:H7 (rEIT), increasing the ELISA method's specificity for specific detection of *E. coli* O157:H7.

Shen et al. (2014) performed an ELISA using immunomagnetic and beacon gold nanoparticles to detect *E. coli* O157:H7. This method had detection limits of 68 CFU mL^{-1} in PBS and 6.8×10^2 to 6.8×10^3 CFU mL^{-1} in the food samples. In Shen et al.'s study, unlike our study, the *E. coli* O157:H7 bacterium was used to produce monoclonal antibodies, but due to the use of immunomagnetic nanoparticles conjugated with monoclonal anti-*E. coli* O157:H7 antibody, the method's sensitivity was higher than other ELISA methods and ours as well. Also, in the current study, the competitive ELISA was done with antigen, *E. coli* O157:H7 bacterium, and mouse antibody. This method had a detection limit of 10^3 CFU/ mL, a relatively more straightforward process than the one for Shen et al. (28).

Pang et al. in 2017, developed a novel paper-based enzyme-linked immunosorbent assay (p-ELISA) for rapid *E. coli* O157:H7 detection. In the study of Pang et al., *E. coli* O157:H7 bacterium was used, which was less sensitive (1×10^4 CFU/ mL.) than the competitive ELISA (10^3 CFU/ mL) in this study, and it had a high specificity similar to our method (29).

In another study, Zhang et al. developed a double antibody sandwich ELISA from intimin gamma 1 (intimin γ1), and one of the most important outer membrane proteins of *E. coli* O157:H7 was used, and the use of intimin γ1 increased the sensitivity and specificity for measuring EHEC O157:H7. In this and our study, the detection limit was 10^3 CFU/ mL for EHEC O157:H7 cultures (30).

CONCLUSION

Our study and previous studies show that ELISA assay is accurate, sensitive, easy, inexpensive, and effective for detecting *E. coli* O157: H7.

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