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ORIGINAL ARTICLE

A rapid and specific PCR–ELISA for detecting Salmonella typhi

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ABSTRACT

Background: Salmonella continues to be a major food borne pathogen for animals and humans. A sensitive and specific PCR–ELISA technique was developed to detect Salmonella typhi.

Materials and methods: The assay was based on the incorporation of digoxigenin-labeled dUTP and a biotin-labeled primers specific for rfbE gene during PCR amplification. The labeled PCR products were bound to streptoavidin-coated wells of a microtiter plate and detected by ELISA. The specificity of the PCR was determined using 4 strains of Entrobacteriaceae family including Escherichia coli, Klebsiella, Proteus, and Enterobacter and 2 strains of salmonella genus including paratyphi A and entritidis.

Results: Among all the strains, only Salmonella typhi was positive. The PCR-ELISA detecting system was able to increase the sensitivity of assay up to 100 fold, compared with a conventional PCR. The detection limit in PCR-ELISA was 2.5ρg in genomic DNA and 20 cells in direct manner per reaction. The entire procedure took about 100 minutes. For further confirmation of the test, internal biotin labeled probe was designed for rfbE gene and detected with streptavidin.

Conclusion: We have developed a rapid and simple PCR-ELISA protocol suitable for routine analysis of viable Salmonella typhi.

Keywords: *PCR-ELISA, Rapid detection, Salmonella typhi, rfbE gene*.

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INTRODUCTION

Salmonella continues to be a major food borne pathogen for animals and humans (1,2) while it is the leading cause of food borne outbreaks and infections worldwide (3-5). Humans are infected through eating raw or undercooked foods, including meat, poultry, egg and dairy products (6). Globally, foods are common sources of microbial infections (7). Therefore, the availability of reliable, rapid and internationally approved test systems to detect food borne pathogens is becoming increasingly important for the food industry and legislative control as well (3,8,9). Standard methods for detecting Salmonella spp. , such as ISO 6579, are time consuming (4–5 days) and involving a pre-enrichment in buffered peptone water (BPW) followed by plating on selective agar (5,10-12). Recently, nucleic acid amplification technologies have offered the potential for improved detection of Salmonella in the environment, providing greater sensitivity and dramatically speeding up detection, thereby improving the management of outbreaks through

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more rapid confirmation of the vehicle of infection (5,11-14).

Molecular techniques such as PCR (polymerase chain reaction) have proven to be specific and sensitive methods for detecting infectious pathogens (7,12,15,16). Although PCR tests were considered attractive, traditional PCR methods required amplification in a thermocycler, and amplification product separation by gel electrophoresis followed by hybridization with a probe. This time-consuming procedure is gradually being replaced by PCR-enzyme-linked immunosorbent assay (PCR-ELISA), which is more convenient for rapid and reliable detection and quantification of pathogen-specific gene sequences (6,17,18). Thus, in vitro amplification of DNA by PCR has now become a potentially powerful alternative in microbiological diagnostics due to its rapidity and accuracy. A digoxigenin (DIG)-ELISA kit manufactured by Roche Diagnostics Corporation (Indianapolis, IN) provides a convenient, nonradioactive detection solution for PCR products in a microtiter plate format. A biotin-labeled primer is used together with DIG-11'-dUTP, and they are both incorporated into PCR products during amplification. The amplified products are immobilized into the streptavidin-coated surface of a microtiter plate via the strong affinity of the avidin–biotin interaction, and then the amplicons are detected with an anti-DIG–peroxidase conjugate through the substrate 2,2'- azino-di-3-ethylbenzthiazoline sulfonate (17,18).

The present study was conducted to assess PCR-ELISA as an important tool for rapid detection of Salmonella typhi.

PATIENTS and METHODS

Bacterial strains and cultivation

Bacterial strains were obtained from Reference Laboratory in Boo-Ali Hospital in Tehran. Three standard strains of Salmonella genus including typhi, paratyphi A and entritidis, as well as 10 strains of Salmonella typhi isolated from blood cultures and 4 strains of Enterobacteriaceae family including E. coli, Klebsiella, Proteus and Enterobacter were used. Bacteria were routinely cultured on tryptic soy broth or agar (Difco, Detroit, MI). Pure strains of Salmonella typhi were inoculated into a culture bottle containing 16ml of trypticase soy broth (Difco) with 0.02% SPS (sodium polyethanol sulfonate), and incubated at 37ºC for 24 hours. Then, biochemical identification was achieved by conventional procedures, and confirmation was carried out by PCR.

Extraction of DNA from bacterial cultures

Genomic DNA of bacteria was prepared by a modified method. Briefly, 5ml of an overnight culture grown in LB broth was harvested by centrifugation. The pellet was resuspended in lysozyme solution (1mg lysozyme ml^{-1} in 0.15M NaCl, 0.1M EDTA, pH 8.0), followed by lysis using 1% SDS, 0.1M NaCl, 0.1M Tris/HCl (pH 8.0) at 60°C. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) in the presence of 5M sodium perchlorate. A 1/10 volume of 3M sodium acetate and 2 volumes of absolute ethanol were added and the nucleic acid was then pelleted by centrifugation, washed with 70% ethanol and dried under vacuum. The DNA pellet was resuspended in TE buffer (10mM Tris/HCl, pH 7.5, 0.1mM EDTA) (19). DNA concentration was obtained by reading OD (optical density) at 260 nm.

Bacterial cell dilutions

An overnight culture of Salmonella typhi was serially diluted (10-fold) with brain heart infusion (BHI) broth. A 100µl aliquot of each dilution was boiled for 10 min, snap-cooled and then centrifuged for 1 min at 13000 rpm. A 4µl aliquot of the supernatant was used as template in the PCR. Viable counts were obtained by plating 100µl of each dilution of bacterial culture on LB plates and incubating overnight at 37°C (13,19).

Table 1. *PCR primers and hybridization capture probe for rfbE gene*

Oligoneucleotide	Sequence(5´-3´)	Nucleotide position Accession no: AF332602	Expect product size(bp)
BS1(forward primer)	GAGGAAGGGAAATGAAGCTTTT	209-230	615 bp
BS2(reverse primer)	TAGCAAACTGTCTCCCACCATAC	824-802	
SS1(probe)	GATATGATGAGAGCACACAATTAGAT	671-696	-

Target gene, PCR primers and reaction conditions

The gene encoding CDP-tyvelose epimerase (rfbE), that is specific for typhoid fever agent, was selected as the target of the assay. Complete sequence of the rfbE gene was obtained from GenBank (accession number AF332602). The DNASIS and OLIGO 5 soft wares were used for comparative analyses and the final design of PCR primers and the nucleic-acid sequences probe. The primers, which promote the amplification of a 615bp fragment, and the DNA probe, were tested using molecular biology software. The oligonucleotides, specific for Salmonella typhi, and the DNA probe used in this study are presented in table 1. The oligoneucleotides were synthesized by Life Technologies (Gaithersburg, MD).

PCR assay

The PCR assay was performed in a final reaction volume of 50µl. Each reaction mixture consisted of 0.3µM of each primer, 200 µM of each dATP, dCTP, and dGTP, 190µM dTTP, 10µM DIG-11-dUTP(Roche Diagnostics), 0.5U of Taq DNA polymerase, 5µl of 10xPCR buffer, and 1.5mM $MgCl_2$ in the presence of different concentrations of genomic DNA (50ng to 0.5pg). The same reactions were conducted using lysed cells as a DNA source (1 to 1500 cell in each reaction).

The PCR program was: 94ºC for 5 min, followed by 5, 8 or 30 cycles at 94, 60 and 72ºC each for 1 min and the final extension at 72ºC for 5 minutes. Then, 5µl of each PCR product was loaded into a well of a 2% agarose gel containing 0.5µg/ml ethidium bromide. A 100bp ladder plus (Fermentas) was used as the molecular weight marker. PCR products were electrophoresed and visualized under UV light, and gel images were stored using a gel documentation system (Gel Doc 1000; BioRad, Hercules, CA).

Detection of labelled PCR production microtitre plates

The detection of the DIG-labelled PCR products was made by using the PCR-ELISA,DIG-Detection Kit (Boehringer Mannheim, Germany). 10µl of PCR amplified product with AH primer pairs added to 90µl of PBS (pH=7.2) and serially diluted in the wells of the microtiter plate and incubated at 65ºC without shaking for 1.5 h until dried. Plates were washed five times with PBS (pH 7.2) containing 0.05% Tween 20 (PBST). 100µl of antidigoxigenin Fab-peroxidase conjugate (Roche Diagnostics) labeled with Horse Radish Peroxidase (HRP) diluted 1:2500 in PBST was added to each well and incubated at 37°C with shaking for 30 min. After washing, 100µl of substrate solution (O-Phenylene Diamine 0.2mg/ml of citrate phosphate buffer, pH 5 containing 500µl 30% H_2O_2) was added in each well of microplate and incubated in the dark at room temperature without shaking for 15 min. 100µl of 1M H_2SO_4 was then added to stop the reaction. The OD was measured at 490 nm using an ELISA reader (Dynex Technologies, Guornesey, Channel Islands, Great Britain) (17,18).

Hybridization and development of the probe

In order to confirm the results, we designed a biotin labeled probe for the middle part of the rfbE gene (table 1). For the hybridization reaction, 80µl of hybridization solution (1×SSC with 0.6pmol biotinylated probe) was added to 20µl of the PCR product of BS primer pairs and denatured by heating for 15 min at 95ºC. 100µl of the hybridization reaction was incubated on ice for 5min; then transferred to the microplate wells sensitized with streptoavidin as described previously. The hybridization was carried out for 3 hours at 55°C. Anti-DIG antibody–peroxidase conjugate (diluted 1:2500 in PBST) was added to each well and incubated at 37°C for 1 hour before the wells were washed five times with the wash buffer. Finally, the peroxidase substrate was added to wells and OD values were recorded with an ELISA plate reader (λ=490 nm) after 15 min of incubation at room temperature (17,18).

RESULTS

Detection of Salmonella typhi by standard PCR

The rfbE gene was chosen as a target for detecting the Salmonella typhi. Alternative conditions for PCR amplification were tested to include higher and lower annealing temperatures as well as higher primer concentrations. The aforementioned conditions were, therefore, found to be the optimum reaction conditions. Standard amplification with the primer pair resulted in a 615-bp band, as seen in the EtdBr stained agarose gel (Fig.1).

Sensitivity of PCR product using genomic DNA and cell lysate as a template

The result obtained from PCR with decimal dilutions of genomic DNA of Salmonella typhi is shown in figure 2. The minimum amount of genomic DNA of Salmonella typhi that produce a visible band on ethidium bromide–stained agarose gel electrophoresis was 25 pg of genomic DNA and was 500 bacterial cells with 30 cycle. Meanwhile, the results of PCR product with 5 cycles were similar to that of 30 cycles (data not shown).

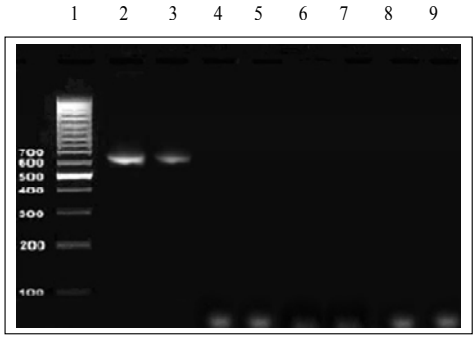

Figure 1. *Detection of* Salmonella *typhi by standard PCR (Lane 1: DNA marker (100bp DNA ladder plus). Lanes 2,3: Salmonella typhi. Lane4: Salmonella paratyphi A. Lane 5: Salmonella entriidis. Lane 6: E.coli. Lane 7: Klebsiella. Lane 8: Proteuse. Lane 9: Entrobacter*.

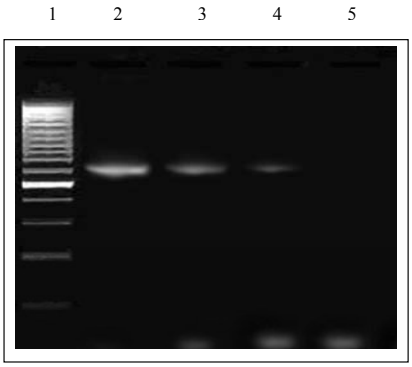

Figure 2. *Detection sensitivity of PCR using genomic DNA, followed by agarose gel electrophoresis (Lane 1: DNA marker (100bp DNA ladder plus). Lane 2-5: The serial dilution (25, 2.5, 2.5×10^{-1}, 2.5×10^{-2}ng) of genomic DNA of Salmonella typhi with 30 cycle).*

Sensitivity of PCR-ELISA assay

This technique determined the lowest level of typhoid fever agent genomic DNA and contamination that could be detected in pure culture by PCR-ELISA. The study was carried out on 12 serial dilution from genomic DNA (table2), and diluted cells culture 5-fold from 10^5 to 1 (table3). Results revealed that the restriction limit of the technique used for the detection of Salmonella typhi is 2.5pg from DNA and 20 bacteria for two primer pairs of culture sample.

Table 2. *Determining the sensitivity of the PCR-ELISA technique against samples derived from serial dilution of DNA extraction of Salmonella typhi and other bacteria*

Dilution	O.D (8cycle)	O.D (5cycle)	DNA quantities
>1/10	>3.000	>3.000	>20(pg)
1/100	2.745	1.153	20(pg)
1/200	2.534	0.982	10(pg)
1/400	1.617	0.346	5(pg)
1/800	0.538	0.187	2.5(pg)
1/1600	0.074	0.056	1.25(pg)
Salmonella partyphi A	0.034	0.028	1.25(pg)
Salmonella entritidis	0.045	0.055	50(ng)
E.coli	0.035	0.036	50(ng)
Enterobacter	0.062	0.025	50(ng)
Klebsiella	0.048	0.063	50(ng)
Proteuse	0.039	0.043	50(ng)
Negative control	0.081	0.067	-

O.D.: Optical density

Table 3. *Determining the sensitivity of PCR-ELISA technique in cultured samples of Salmonella typhi*

Cell number	O.D (8cycle)	O.D (5cycle)
1000	>3.000	3.455
750	3.068	2.931
500	2.530	1.686
250	1.254	0.810
100	0.500	0.182
20	0.176	0.037
5	0.049	0.044
1	0.049	0.038
Negative control	0.038	0.039

O.D.: Optical density

Sensitivity of detection in hybridization

In order to confirm the PCR products of rfbE gene of Salmonella typhi and also determine the minimum concentration of DNA that could be detected, the hybridization assay was achieved. Results were positive and demonstrated the detection limit. Table 4 represents the sensitivity of hybridization according to the serial dilution from genomic DNA.

Specificity of PCR-ELISA technique

The specificity of the PCR-ELISA was assessed on 17 strains comprising 10 strains of Salmonella typhi currently found in blood samples, 3 standard strains of Salmonella genus including typhi, paratyphi A and entritidis, and 4 strains of Enterobacteiaceae family including E. coli, Klebsiella, and Proteuse. The absorbance readings of 11 samples of Salmonella typhi were higher than the cut-off point, whereas the absorbance readings of the others bacteria were lower than 0.052 in PCR-ELISA, indicating that the PCR-ELISA test was suitably reliable.

Table 4. *Results of optical density of hybridization with serial dilution from genomic DNA at the end of PCR cycle*

Dilution	O.D (8cycle)	DNA quantities
>1/10	>3.000	40(ng)
1/100	2.152	4(ng)
1/200	1.967	2(ng)
1/400	1.548	1(ng)
1/800	1.372	100(pg)
1/1600	1.087	50(pg)
1/3200	0.590	25(pg)
1/6400	0.309	12.5(pg)
1/128000	0.192	6.26(pg)
1/256000	0.114	3.12(pg)
1/512000	0.090	1.56(pg)
Salmonella partyphi A	0.041	50(ng)
Salmonella entritidis	0.052	50(ng)
E.coli	0.046	50(ng)
Enterobacter	0.031	50(ng)
Klebsiella	0.039	50(ng)
Proteuse	0.052	50(ng)
Negative control	0.046	-

O.D.: Optical density

DISCUSSION

Salmonella is a facultative, intracellular parasite that invades the mucous membrane, and is transmitted to humans mainly through water, meat, eggs and poultry products (1,8). Salmonella infection is the most frequent food-borne gastrointestinal disease transmitted from animals to humans (2,3). Typhoid fever still remains endemic in many developing countries. Good monitoring and screening programs are required in order to prevent salmonella infections (4,5,8). Detection of Salmonella by bacteriological methods is a time-consuming procedure that usually requires 5–11

days (5,8). Furthermore, increased public awareness related to the health and economic impact of food-borne contamination and illness has resulted in greater efforts to develop more sensitive methods of pathogenic detection and identification. Therefore, efforts have been made by prior investigators to reduce the required time and increase the sensitivity of detection technique (5,11,13).

During the recent years, numerous molecular diagnostic approaches have been developed for detecting and analyzing bacterial strains, including various PCR assays (5,11,17). While most of the PCR conditions were already established, several optimization procedures for the DIG-ELISA were studied, including using various coating concentrations of streptavidin, adding various amounts of PCR products to the streptavidin-coated microplate, and removing excess free primers and nucleotides from the PCR products by a chromatography method (Wizard DNA Clean-up system; Promega, Madison, Wis.) prior to addition to the streptavidin plate. PCR-ELISA method is faster and provides greater sensitivity, therefore, it improves the management of outbreaks compared to bacteriological techniques.

Our findings indicate that this approach could become an international standard and could be employed with confidence in microbiologic laboratories. The conventional PCR assay is already an established laboratory technique, but adaptation of the test in a PCR-ELISA format represents a further development of the method, providing highly specific results in a shorter time by using biotin-label primers which improve the specificity of the assay. The advantage of the DIG-ELISA is the simplicity of the method, together with the solid-phase 96-well (or even 384-well) microplate format, which allows many samples to be analyzed at one time. Automation of the PCR and ELISA procedures with robotic equipment also makes large-scale screening of samples possible (6,17,18). It is conceivable that hundreds of samples could be screened and proven to be free of salmonellae within 48 hours, and in the meantime, for those positive samples that are found, another day or two would be needed for serotype identification.

In attempts to improve the sensitivity of the PCR assay for the detection of salmonellae, we developed a DIG-ELISA to further enhance the detection level of amplified PCR products.

In this paper, PCR-ELISA correctly detected a specific sequence of the rfbE gene of typhoid fever agent. The fact of non-positive results of the other strains indicates that the tests are highly specific. Additionally, the PCR-ELISA tests proved to be highly sensitive, able to detect 20 cells. The specificity of the primers was determined using 7 bacterial strains, among which only the typhoid fever agent was amplified. Moreover, we have compared the performance of PCR-ELISA with standard agarose gel electrophoresis. ELISA assay was 100-fold more sensitive than the gel electrophoresis.

In summary, the PCR–ELISA assay is quite sensitive, specific, and rapid for detection of Salmonella typhi. Automation of the PCR and ELISA procedures with robotic equipment will enable large-scale screening of typhoid fever agent.

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