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Original Article

Evaluation of Multiplex PCR System for Simultaneous Detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enteritidis* in Shrimp Samples

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A multiplex polymerase chain reaction (PCR) method was evaluated for simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enteritidis* in shrimp samples. The sensitivity of DNA amplification by PCR in this method was found to be 10^3 cfu/ml for each pathogen. When this protocol was adopted for the detection of each of the above mentioned pathogen in spiked shrimp extract culture, similar sensitivity was observed. However, this method detected 1 bacterial cell for *E. coli* O157:H7 and *S. enteritidis* and 100 for *L. monocytogenes* per 25 g spiked shrimp samples after overnight enrichment. In the commercially imported shrimp samples, none was found to contain any of the three pathogens by multiplex PCR or by conventional method, which suggests that the multiplex PCR is a reliable and useful for rapid screening of shrimp samples for *E. coli* O157:H7, *L. monocytogenes* and *S. enteritidis.* This will save time and increase our ability to assure food safety.

Keywords: Multiples PCR, Shrimp extract, Spiked sample, *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enteritidis*

Introduction

Food borne pathogens remain a major public health concern worldwide. Outbreaks of human illness associated with the consumption of fresh fruits and vegetables have been reported from many countries[1-2]. *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. are considered as major food-borne pathogens. An increased number of these bacterial infections associated with the consumption of fresh produce, such as seafood, raw meats, and ready-to-eat foods in which conventional thermal pasteurization and sterilization are not easily applicable to inactivate microorganisms, have been documented in recent years[3]. Several major outbreaks of *E. coli* O157:H7 has been demonstrated, and most incidences have been associated with the consumption of contaminated ground beef, milk, water, and dairy products[4]. *L. monocytogenes*, being present in soil and other natural environment can contaminate plant and animal products easily[1,5]. Milk, cheese, ice cream, meats, and ready-to-eat foods have been reported as major contamination sources for human listeriosis[6-7]. Furthermore, its ability to grow at refrigeration temperatures, and survive within biofilms, imposes an additional risk. The importance of *Salmonella* as a food-borne pathogen is well established as it occurs in poultry and poultry products as well as in raw meats, milk and other dairy[8-9].

Reliable detection techniques with easiness, rapidity and high sensitivity are prerequisite for identification of these pathogenic bacteria in foods, food sources, and food processing plants. Since conventional culture methods for detection of pathogens are time consuming, these methods take several days to make the results available, thus increasing the risk of uptake or transmitting pathogens. Pathogens are often present in very low numbers with a high background of indigenous microflora, thus rendering recovery of target organisms difficult. Advances in modern biotechnology have enabled us to use more reliable and sensitive identification systems. PCR-based methods have potential for rapid and sensitive detection of food borne pathogens. Recently, a number of PCR-based detection methods have been investigated for *E. coli* O157:H7, *L. monocytogenes*, *S. enteritidis*[10].

The reliability of PCR-based detection methods partly depends on the target bacterial cell number, *i.e*., the copy numbers of target molecules present in food samples. An enrichment culture system is often needed to overcome the problems of low pathogen numbers. Furthermore, several studies have reported to use of multiplex PCR systems to detect two or more pathogens in one assay. A combination of immunomagnetic separation (IMS) method with a multiplex PCR system for simultaneous detection of *E. coli* O157:H7, *L. monocytogenes, Campylobacte*r and

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Salmonella spp. in milk has been reported[11]. Recently, a multiplex PCR system has been developed for simultaneous detection of these three pathogens in meat samples in food hygiene team, National Food Research Institute. DNA detection sensitivity for this method is 10^3 cfu/ml for each pathogen. In spiked pork samples, 1 cell per 25 g of inoculated sample could be detected within 30 h for each pathogenic bacterium. Moreover this multiplex PCR system was confirmed to be able to detect these pathogenic bacteria with compatible or superior sensitivity to the conventional method over the same time period in the samples of naturally contaminated meat[12].

In this study, we investigated the detection sensitivity of the multiple PCR system when applied for frozen shrimp that are mainly produced for export markets in South Asia including Bangladesh. We also evaluated the optimization of the pre-enrichment conditions, the DNA extraction method, and the multiplex PCR settings.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this experiment were from our laboratory collection, which included *L. monocytogenes* strains ATCC 43256 and ATCC 49594, *S. enteritidis* IFO 3313 and *E. coli* O157:H7. The strains were maintained tryptic soy agar (TSA; Nissui Seiaku Ltd., Tokyo, Japan) slants at 4ºC. Bacterial stains were usually grown in trypticase soy broth (TSB; BBL, Becton Dickinson, Sparks, USA) at 37ºC overnight. For viable cell determination, serial decimal dilutions of samples with phosphate buffered saline (PBS, pH 7.2) were made and plated on deoxycholate hydrogen sulphide lactose agar (Eiken Co., Ltd., Tokyo, Japan) for *Salmonella*, on Oxoid modified agar (Oxoid, Hampshire, UK) for *L. monocytogenes*, and on modified *E. coli* agar (Eiken Co., Ltd., Tokyo, Japan) for *E. coli* O157:H7. The plates were then inoculated at 37ºC for 24 to 48 h before the colonies were counted.

Multiplex PCR settings

PCR was performed in a total volume of 50 µl containing 2 µl of template DNA and 48 µl of PCR master mix composed of 1x PCR buffer, 5.0 m*M* $MgCl_2$, 80 n*M* of each of *E. coli* O157:H7 detection primers (VS-8: 5'-GGCGGATTAGACTTCGGCTA-3'; VS-9: 5'-CGTTTTGGCAGTATTTGCCC-3'), 100 n*M* concentration of each *of L. monocytogenes* detection primers (LM-1: 5'-CGGAGGTTCCGCAAAAGATG-3'; LM-2: 5'-CCTCCAGAGTGATCGATGTT-3'), and 120 n*M* concentration of each of *Salmonella* detection primers (TS-5: 5'-GTCACGGAAGAAGAGAATCCGTACG-3'; TS-11: 5'-GGAGTCCAGGTTGACGGAAAATTT-3'), 200 µ*M* dATP, dCTP, and dGTP, 400 µ*M* dUTP, 0.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Foster City, USA), and 0.5 U of AmpErase UNG (uracil-N-glycosidase; Applied Biosystems Foster City, USA) with a DNA thermal cycler (GeneAmp PCR system 9700, Applied Biosystems Foster City, USA). The thermocycler was programmed as 50ºC for 2 min for carryover treatment, and initial denaturation at 95ºC for 10 min. The samples were then subjected to 40 cycles of 95ºC for 20 sec, 60ºC for 30 sec 72ºC for 30 sec, and then 72ºC for 7 min. The amplified products were then analyzed by 2.5% agarose gel electrophoresis. Expected size for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* were 120, 234 and 375 bp, respectively.

DNA extraction method

For extraction of DNA in food samples, guanidine isothiocyanate (GuSCN) lysis method which previously developed for multiplex PCR system was used[12]. First the bacterial cells were resuspended in 200 µl of digestion solution containing 1 mg/ml acromopeptidase and 1 mg/ml lysozyme in 10 m*M* Tris/HCl-1 m*M* EDTA (TE) buffer, pH 8.0. After incubation for 1 h at 37ºC, the solution was mixed with 300 µl of 4 *M* GuSCN containing 2% Tween 20. The solution was spun for 5 min at 15,000x *g* and 400 µl of the supernatant was transferred to a new tube containing 400 µl of 100% isopropanol. The mixture was then centrifuged for 15 min at 15,000x *g*, and the resulting pellet was washed with 70% isopropanol. The DNA pellet was dried briefly and dissolved in 160 µl of deionised water and heated for 70ºC for 3 min prior to use, the template DNA solution was centrifuged for 5 min at 15,000x *g* to remove water insoluble impurities. Two microlitres of the solution was used as a PCR template.

Optimization of multiplex PCR

Optimization of the multiplex PCR system for simultaneous detection of *E. coli* O157:H7, *L. monocytogenes* and *S. enteritidis* were done using DNA from pure culture for each organism. Each pathogen was grown on TSB at 37ºC overnight and a serial decimal dilution as made with PBS. One millilitre of each dilution was used for DNA preparation by above mentioned method.

Multiple PCR evaluated with the mixed culture

The sensitivity of the multiplex PCR system for simultaneous detection of *E. coli* O157:H7, *L. monocytogenes*, and *S. enteritidis* were evaluated with the mixed cultures prepared as follows. Each pathogen was grown to the late logarithmic phase in No. 17 medium. The cells were collected by centrifugation and resuspended in the original volume of PBS. Serial decimal dilutions were made with PBS. One millilitre of each dilution was mixed with 9 ml of shrimp culture extract (25 g of shrimp was stomached with 225 ml of No. 17 broth and incubated at 35ºC for 24 h) and immediately proceeds for DNA isolation by lysis-GuSCN method. Shrimp samples that were confirmed as negative for the experimental pathogens were employed.

Evaluation of multiplex PCR with the inoculated shrimp samples

One millilitre of a mixed culture containing *S. enteritidis*, *L. monocytogenes* and *E. coli* O157:H7 containing a concentration of 0-10^2 cfu was mixed with 25 g of shrimp; 225 ml of No. 17 broth were added to the sample and stomached for 2 min. The resulting mixture was then incubated at 35ºC for 24 h. After 24 h, 1 ml of broth was used for DNA extraction by lysis-GuSCN method and subjected to the PCR assay as described before. When the shrimp with shell samples were used, the modified DNA extraction methods were done as the following. Firstly, a digestion solution

was made in TE containing 10 m*M* EDTA. Secondly, a ten times higher concentrations of acromopeptidase (10 mg/ml) in normal TE was used. Thirdly, the concentration of lysozyme was increased up to 10 mg/ml. Lastly the mixed culture was passed through 5, 30, 70 and 100 µm pore sizes of filters followed by DNA extraction.

Multiplex PCR experiment in actual shrimp samples

Nearly, 20 frozen shrimp samples imported from Bangladesh were purchased from the domestic retailer. The samples were kept at -20ºC freezer until analyzed. Twenty five grams of shrimp from each sample was mixed with 225 ml of No. 17 broth, stomached, and incubated for 35ºC for 24 h, and then 1 ml of culture broth was used for DNA isolation.

Results and Discussion

Optimization of the PCR assay

Each primer pair used in this study was selected carefully to avoid cross reaction. The primer pairs selected in this study were TS-5 and TS-11 for amplification of a 375-bp sequence from the *Salmonella*-specific DNA sequence[13], LM-1 and LM-2 for amplification of a 234-bp sequence from the haemolysin (*hly*A) gene of *L. monocytogenes*[14], and VS-8 and VS-9 for amplification of a 120-bp sequence from the *eae*A gene of *E. coli* O157:H7[15]. The PCR method was optimized by adjusting the concentration of primer pairs with purified DNA from each pathogen (Figure 1). The specificity was performed by using DNA from non-target organisms (data not shown).

Sensitivity of multiplex PCR system

According to the established multiplex PCR system, the detection limit of individual target pathogen was estimated to be 10 cells per reaction tube; which represented 10^3 cfu/ml of culture. When the same protocol was tested with the pure cultures of *E. coli* O157:H7, *L. monocytogenes* and *S. enteritidis*, we achieved the same level of sensitivity for the detection of each pathogen as previously reported. The sensitivity was also determined with

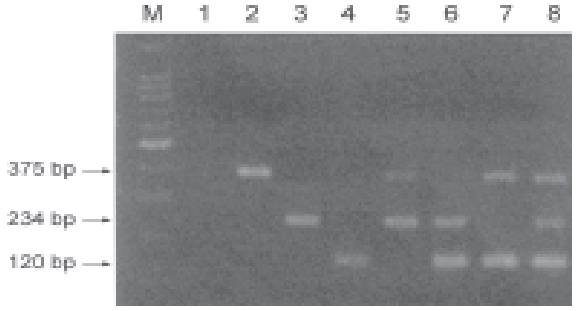

Figure 1. *Multiplex PCR detection of Escherichia coli O157:H7, Listeria monocytogenes, and Salmonella enteritidis. Lane 1: negative control; lane 2: 200 fg of S. enteritidis; lane 3, 200 fg of L. monocytogenes; lane 4: 200 fg of E. coli O157:H7 DNA per reaction tube; lane 5: 200 fg each of L. monocytogenes and S. enteritidis; lane 6: 200 fg each of E. coli O157:H7 and L. monocytogenes; lane 7: 200fg each of E. coli O157:H7 and S. enteritidis; lane 8: 200 fg each of E. coli O157:H7, L. monocytogenes and S. enteritidis DNA per reaction tube.*

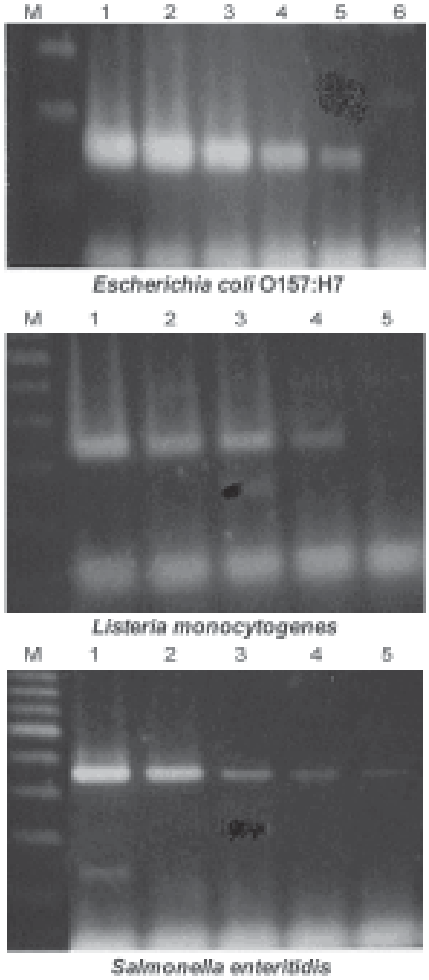

Figure 2. *Sensitivity of multiplex PCR method. DNAs were prepared from Escherichia coli O157:H7, Listeria monocytogenes and Salmonella enteritidis. PCR amplification was done with 3 pairs of primers and template DNAs were used as 2 ng (lane 1), 200 pg (lane 2), 20 pg (lane 3), 2 pg (lane 4), 200 fg (lane 5) and 20 fg (lane 6).*

the purified genomic DNA of all three pathogenic bacteria. In all cases, the lowest limit of detection was observed in 200 fg of DNA in each pathogenic bacterium (Figure 2).

Evaluation of multiplex PCR assay with shrimp extract

The multiplex PCR assay for the detection of *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* has been evaluated in ground pork samples[12]. In the present study, shrimp samples negative for these pathogen was used. In the case of shrimp samples without shell on, all the three pathogens were detectable at a dilution of 10^3 cfu/ ml in extract of shrimp enrichment culture (Table 1, Figure 3). However, when shrimp samples with shell were used, the lowest level of detection was found at a dilution of 10^4 cfu/ ml of shrimp culture. To overcome the problem with shrimp extract with shell, some variation in DNA extraction method was made; and it was found that filtration through 5 µm filter before DNA extraction could achieve similar detection sensitivity to that of shrimp samples without shell on at a dilution

Table 1. *Evaluation of multiplex PCR assay with shrimp extract mixed with different of dilution of each of the pathogen followed by PCR assay*

Shrimp extract inoculated with bacteria	Detection limit (cfu/ml)		
	Escherichia coli O157	*Salmonella enteritidis*	*Listeria monocytogenes*
Shrimp without shell	10^3	10^3	10^3
Shrimp with shell	10^4	10^4	10^4
Shrimp with shell passed through 5 mm filter	ND	ND	10^3
Cells only	10^3	10^3	10^3

ND = Not done

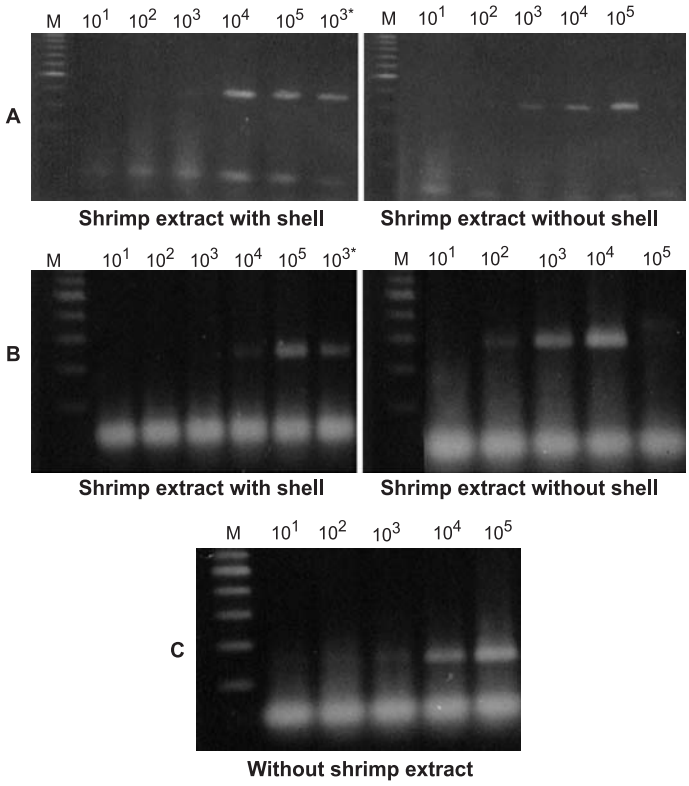

Figure 3. *(A) Detection of Salmonella in different dilution mixed with shrimp extract with and without shell followed by PCR assay. (B) Detection of Listeria monocytogenes in different dilutions mixed with shrimp extract with and without shell followed by PCR assay. (C) Same experiment with Escherichia coli O157:H7 (the result of experiment without shrimp extract was shown). M = Molecular marker ladder; * = When only 10^3/ml cells were used.*

of 10^3 cells per ml. This sensitivity improvement is possibly due to the retainment on the filter of the inhibitory substances of PCR reaction (data not shown).

Evaluation of multiplex PCR assay with the inoculated and actual shrimp samples

The sensitivity of multiplex PCR system was evaluated using shrimp samples inoculated with three pathogenic bacteria at low cell concentrations. In the inoculated shrimp samples, *E. coli* O157:H7 and *S. enteritidis* were both detected as low as 1 cfu per 25 g of shrimp samples after enrichment in No. 17 broth for 24 h (Table 2). However, this method failed to detect *L. monocytogenes* at any culture dilutions tested. *L. monocytogenes* was detectable at a concentration of only 10^2 cells per 25 g of shrimp even when inoculated alone. One of the main reasons might be due to the growth competition among these pathogens in the presence of high back ground natural microflora. The shrimp samples used in this experiment contained approximately 6 log cfu/g natural microflora.

Table 2. *Evaluation of multiplex PCR assay for the detection of Escherichia coli O157, Salmonella enteritidis and Listeria monocytogenes inoculated in shrimp samples and enriched for 24 h followed by DNA extraction by lysis-GuSCN method*

Dilution of pathogen inoculated	Detection result		
	Escherichia coli O157	*Salmonella enteritidis*	*Listeria monocytogenes*
10^2	+	+	+*
10^1	+	+	-
10^0	+	+	-
0	-	-	-

* = Detectable only when inoculated alone

In this study, multiplex PCR assay was also tested for detection of these pathogens in the imported frozen shrimp samples purchased from the domestic retailer. The PCR assay was also compared with the conventional culture method. Of the 20 samples tested, none of them were found to be positive by either of the methods (Table 3).

Table 3. *Comparison of results obtained in multiplex PCR and conventional culture methods in shrimp sample (n = 20)*

Pathogen tested	Conventional culture method	Multiplex PCR method
Eschrichia coli O157	Neagative	Neagative
Salmonella enteritidis	Neagative	Neagative
Listeria monocytogenes	Neagative	Neagative

The main problem still to be solved in this study is detection limit of *L. monocytogenes* when inoculated in lower number along with the other two pathogens. Further study will help resolving this problem.

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