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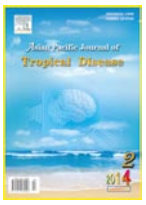

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Development of a multiplex polymerase chain reaction protocol for the simultaneous detection of *Salmonella enterica* serovar Typhi and Class 1 integron

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ABSTRACT

Objective: To develop a multiplex polymerase chain reaction (PCR) protocol for the simultaneous detection of *Salmonella enterica* serovar Typhi (*S.* Typhi) and Class 1 integron, so as to aid rapid diagnosis of *S.* Typhi cases and help in the selection of treatment options based on the presence of the Class 1 integron that can carry resistance cassettes to a range of antibiotics.
Methods: PCR for amplification of specific regions was done using *fliC-d* and *intl* primers and agarose gel electrophoresis was used for resolution of PCR products.
Results: The *fliC-d* primer (*S.* Typhi specific) amplified a 587 bp region and the *intl* primer (Class 1 integron specific) amplified two bands approximately 500 and 550 bps. The developed method was specific for *S.* Typhi and did not amplify any products with *Salmonella enterica* serovar Typhimurium ATCC 14028, *Salmonella enterica* serovar Paratyphi and *Escherichia coli* O157:H7.
Conclusions: The developed multiplex PCR protocol can be used for rapid diagnosis and aid in proper treatment strategies for patients infected with *S.* Typhi.

1. Introduction

Typhoid fever remains a serious public health problem in Southeast Asia. Traditionally, the agent of typhoid fever, *Salmonella enterica* serovar Typhi (*S.* Typhi) is diagnosed using culture methods and biochemical tests which require 4-5 d for completion. The need for the development of rapid, alternative methods for the detection of *S.* Typhi has been recognized in the past. Several studies have reported on the development of rapid polymerase chain reaction (PCR) based methods for the detection of *S.* Typhi and other *Salmonella* serotypes[1-11].

With the rise of multidrug resistant, the need for rapid diagnosis of drug resistant *S.* Typhi cannot be overemphasized. Bacteria dissemination of resistance is determined by mobile elements including plasmids, transposons, and gene cassettes in integrons[12-17]. Among the four distinct classes of integrons encoding different integrases that have been reported[16,18], Class 1 integrons are the most frequent in clinical strains. Class 1 integrons often contain *sul1*, which encodes resistance to sulphonamides[14,18,19]. At present, about 60 different cassettes associated with resistance genes have been identified, and the same cassettes can be found in different classes of integrons[19-21]. A PCR based method for the detection of *S.* Typhi and selected drug resistance (ciprofloxacin, ampicillin, chloramphenicol and cotrimoxazole) has been developed earlier[22]. In this study, we aimed to develop a multiplex PCR method for the detection of *S.* Typhi and Class 1 integron specific genes. This will enable to rapid the detection of the pathogen as well as give an indication of antibiotic resistance gene cassettes presented in the isolates, which will contribute to enable the selection of antimicrobials that are not commonly associated with Class 1 integrons.

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2. Materials and methods

2.1. Bacteria

Reference *S.* Typhi and Paratyphi were collected from the Centre for Advanced Research in Science (CARS), Dhaka University. *Salmonella enterica* serovar Typhimurium ATCC 14028 (*S.* Typhimurium ATCC 14028) was obtained from a renowned local hospital. Two clinical isolates, one *S.* Typhi (S1) and another *S.* Paratyphi (S2), were kindly provided by a local hospital that confirmed the identity of the isolates by biochemical tests and serology.

2.2. Biochemical test

All bacterial strains were reconfirmed by biochemical tests using specific media (Simmons citrate, Kligler iron agar, methyl red test, Voges-Proskauer test, indole test, motility test media purchased from Oxoid, UK).

2.3. Serology

A single bacterial colony was mixed well with a drop of Poly O antisera (Poly O Factors A-S, Mast Assure™, Merseyside, UK) placed on a clean glass slide. After thorough mixing, the slide was observed for visible agglutination/clumping of the bacterial cells.

2.4. PCR

2.4.1. Template DNA

Colony PCR was carried out. A single colony of *S.* Typhi grown on xylose lysine deoxycholate agar medium was transferred using a sterile toothpick into 50 μL distilled water and heated at 99 °C for 10 min. After cell lysis, cell debris was collected at the bottom by brief centrifugation. From the supernatant, 2 μL of sample containing DNA was used as template.

2.4.2. PCR primers

PCR primers used in this study were specific for *fliC-d* gene (*FliC-d* forward 5′-GCT TAA TGT CCA AGA TGC CTA C-3′, *FliC-d* reverse 5′-GAG CAA CGC CAG TAC CAT CTG-3′) and Class I integron, *intl* gene (*Intl* forward 5′-ACA TGT GAT GGC GAC GCA CGA-3′ and *Intl* reverse 5′-ATT TCT GTC CTG GCT GGC GA-3′)[23,24].

2.4.3. Master mix

PCR Master mix (NEB, UK) contained deoxynucleotide triphosphates, *Taq* polymerase, $MgCl_2$ and buffer, and was concentrated twice (2×).

2.4.4. PCR cycle condition (amplification)

PCR reaction mix was prepared by adding 10 μL master mix (2×), 1 μL of each forward and reverse primer (10 μmol/L), 1 μL $MgCl_2$ (15 mmol/L) to 5 μL PCR grade water. PCR reaction mixture (18 μL) was dispensed in each PCR tube containing 2 μL of template DNA to have a final volume of 20 μL. PCR amplification was carried out by initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95° C for 30 seconds, annealing at 45-65 °C for 45 seconds, elongation at 68 °C for 45 seconds and additional elongation at 68 °C for 10 min.

2.5. Resolution of the PCR product

2.5.1. Preparation of agarose gels

The PCR products were analyzed using agarose gel electrophoresis (2%). In order to prepare 2% agarose gel, 2 g of agarose (First base, Singapore) was added to 100 mL 1×Tris-borate-ethylene diamine tetraacetic acid buffer. The melted agarose was allowed to cool to about 50 °C and was poured into a gel electrophoresis unit. The comb was removed carefully after the solidification of the gel.

2.5.2. Loading and running the sample.

A volume of 10 μL of PCR product was mixed with 2 μL of gel loading dye (6×). A 100 bp molecular weight marker (NEB, UK) was loaded alongside the PCR products. Electrophoresis was carried out at 60-70 V for 30-45 min.

2.5.3. Staining and visualization of the gel

After electrophoresis, the gel was stained in ethidium bromide (40 μg/mL) for 15 min. Distilled water was used to destain the gel for 5 min. The bands were visualized and photographed by gel documentation system (Biorad, USA).

2.6. Multiplex PCR

The reaction mix of single plex PCR was used except the 0.5 μL mix of each primer.

2.7. Sequencing of DNA

The amplicon was purified using Invisorb® DNA CleanUp (Stratec, Germany) following the manufacturer's instructions.

2.8. Measurement of DNA concentration

The concentration of DNA was measured using NanoDrop spectrophotometer (ABI, USA) at 260 nm wavelength of light.

A volume of 1 μL purified DNA sample was applied to the NanoDrop machine and the concentration of DNA per micro liter was measured using elution buffer (Invisorb, Stratec, Germany) as blank.

2.9. Sequencing of PCR product

Purified DNA was sequenced at the CARS, Dhaka University.

3. Results

3.1. Biochemical test results

Biochemical test results of the stock cultures were matched with the standard reaction to confirm the identity of the studied pathogenic bacteria (Table 1).

3.2. Serological test

Salmonella specific O antiserum was used to confirm the seropositivity of the stock strains: Laboratory reference strains of S. Typhi and *S.* Paratyphi, *S.* Typhimurium ATCC 14028 and clinical isolates S1 and S2. *Bacillus thuringiensis* (laboratory strain) was used as negative control.

3.3. Optimization of PCR with *fliC–d* and integron Class 1 (*intl1*) primers

Initially, the PCR of *fliC–d* and *intl1* was done separately using reference strain *S.* Typhi from CARS to optimize the PCR conditions for each in turn. PCR primers were used for amplification of *fliC–d* and *intl1*. The *fliC–d* primer was used in earlier detection of *S.* Typhi and the *intl1* primer was used to investigate the spread of Class 1 integrons in *Salmonella* serotypes[23,24]. In each PCR, a range of temperatures from 40 °C to 55 °C was set to determine the best option for primer annealing. ATCC *S.* Typhi showed no band for either *fliC–d* or *intl1* (gel not shown).

In the absence of any band with *S.* Typhi (laboratory referece strain), we used clinical isolates as template for the same PCRs (Figure 1). A band of approximately 585 bp was found for *fliC–d* primer amplified product and two bands of about 550 and 500 bp were found for *intl1* primer amplified product. As the reference *S.* Typhi did not yield any of the PCR amplicons and both of these products were obtained for isolates S1 and S2, we decided to sequence the *fliC–d* product of one of these isolates (S2) and use it as a reference strain in future experiments.

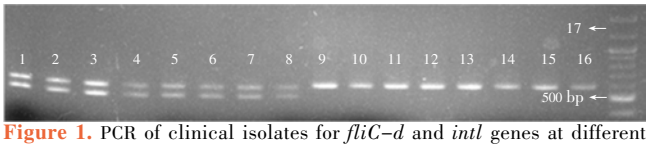

Figure 1. PCR of clinical isolates for *fliC–d* and *intl* genes at different temperatures.
Lane 1–2: S2 55 °C *intl*; Lane 3–4: S2 50 °C *intl*; Lane 5–6: S2 45 °C *intl*; Lane 7–8: S2 40 °C *intl*; Lane 9–10: S2 55 °C *fliC–d*; Lane 11–12: S2 55 °C *fliC–d*; Lane 13–14: S2 55 °C *fliC–d*; Lane 15–16: S2 55 °C *fliC–d*; Lane 17: Ladder 100 bp.

3.4. Increased specificity of PCR by addition of dimethylsulfoxide (DMSO)

We proceeded to investigate the occurrence of two bands with the integron primer. Initially it was assumed that nonspecific band appeared owing to lack of stringency. We decided to include DMSO in the reaction mixture to increase the specificity of the PCR reaction. Two bands were still obtained (Figure 2).

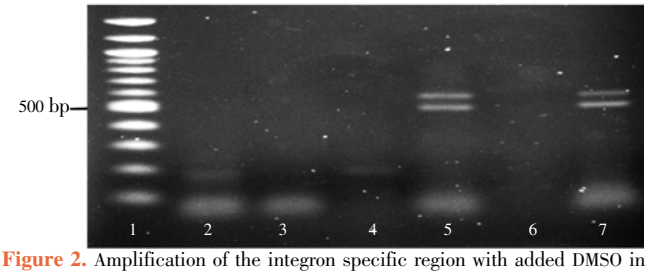

Figure 2. Amplification of the integron specific region with added DMSO in the PCR reaction mixture.
Lane 1: Ladder 100 bp; Lane 2: *S.* Typhi (laboratory referece strain); Lane 3: *S.* Paratyphi (laboratory referece strain); Lane 4: ATCC *E. coli* O157:H7; Lane 5: S2 55°C; Lane 6: Blank; Lane 7: S2 55°C.

3.5. Optimization of PCR with higher annealing temperature and DMSO

During optimization of PCR, DMSO was used to inhibit nonspecific band formation. This still yielded two bands. To rule out the possibility of non-specific band formation, the annealing temperature was raised. Temperatures of 60 °C and

Table 1
Biochemical reaction profile of the bacteria.

Bacteria	Simmon's citrate agar	Kligler's iron agar	Methyl red test	Voges- Proskauer test	Indole test	Motility
Reference *S.* Typhi	+ve	H_2S, sucrose and gas production+ve, lactose-ve	+ve	-ve	-ve	+ve
Reference *S.* Paratyphi	+ve	H_2S, sucrose and gas production+ve, lactose-ve	+ve	-ve	-ve	+ve
S. Typhimurium ATCC 14028	+ve	H_2S, sucrose and gas production+ve, lactose-ve	+ve	-ve	-ve	+ve
E. coli O157:H7	+ve	H_2S, sucrose, gas production+ve	+ve	-ve	+ve	+ve
Clinical isolate No. 2	-ve	H_2S production and sucrose+ve, lactose-ve,	+ve	-ve	-ve	+ve

E. coli O157:H7: *Escherichia coli* O157:H7.

65 °C were used (Figure 3). Two bands were still observed at the higher annealing temperatures.

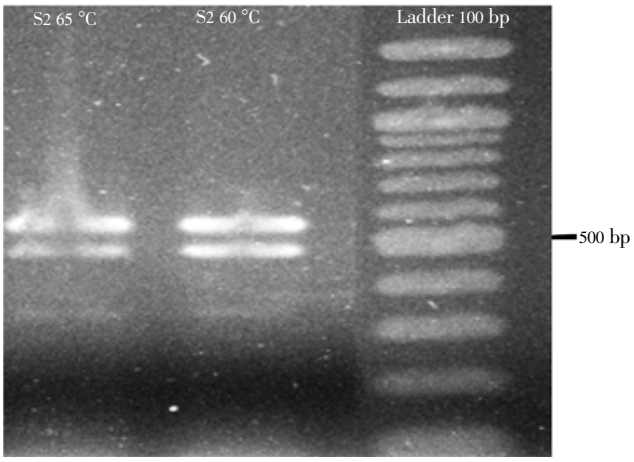

Figure 3. Amplification of PCR products at higher annealing temperatures of 60 °C and 65 °C.

3.6. Multiplex PCR analysis

Multiplex PCR was set at annealing temperatures of 60 °C and 65 °C (Figure 4). The gel picture shows that the clinical isolate S2 gave band with *fliC-d* of 587 bp and integron yielded two bands of sizes 550 and 500 bp.

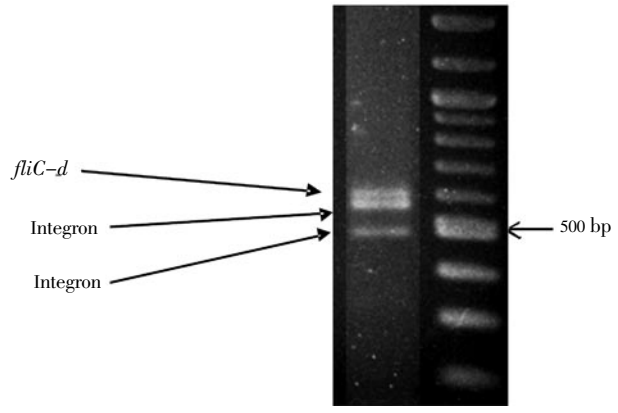

Figure 4. Multiplex PCR to simultaneously detect *fliC-d* and *intl* genes.

3.7.Sequencing of fliC-d PCR product.

The *fliC-d* PCR product was confirmed to be that specific for *S.* Typhi by sequencing the amplified DNA.

4. Discussion

Typhoid fever continues to be a major health problem in many parts of the world particularly in the developing countries. As most of the people here live below the poverty line in unhygienic crowded areas and they hardly have any knowledge on sanitation. They do not maintain hygienic condition during food preparation and handling. As a result typhoid fever affects many people in this country every year. Children and elder people are mostly affected by the notorious organism *S.* Typhi. Diagnosis of Typhoid fever is a lengthy process and sometimes false positives or false negative results are obtained. The need for a more specific and reliable method for identification of *S.* Typhi cannot be overemphasized. Fortunately, with the advent of molecular methods like PCR, diagnosis of different infectious diseases has become easier and more rapid.

There are several reports in the existing literature concerning the identification of different *Salmonella* serotypes including *S.* Typhi[1-11]. However, limited studies have been conducted to identify drug resistance pattern in the causative agents. In the present investigation, a multiplex PCR assay was developed for the detection of *S.* Typhi and Class 1 integron. Multiplex PCR analysis involves using more than one set of primers. Gene *fliC-d* is a flageller gene conserved for *S.* Typhi[1,23]. Class 1 integron contains antibiotic resistance genes. Integrons in *S.* Typhi have been implicated in carrying resistance to ampicillin, chloramphenicol, tetracycline, trimethoprim, and sulphonamides[20]. The objective of the present study was to develop a method that can detect *S.* Typhi and Class 1 integron at the same time. This has a great significance in clinical microbiology if it detects *S.* Typhi and the drug resistance gene at the same time. This would aid treatment options administered to patients infected with the typhoid bacteria. Previous studies have mainly concentrated on the identification of different *Salmonella* serotypes.

We have optimized the conditions for the simultaneous amplification of the *fliC-d* and *intl* regions of *S.* Typhi. In previous studies, multiplex PCR for the detection of S. Typhi and Paratyphi using *tyv* (rfbE), *prt* (rfbS), *viaB*, and fliC genes has been reported[1]. Other workers have documented the detection of *S* Typhi by selective amplification of *invA*, *viaB*, *fliC-d* and *prt* genes by multiplex PCR[23]. But to our knowledge, this is the first report on the detection of *S.* Typhi and Class 1 integron by multiplex PCR. In our PCR, we detected two amplicons specific for the *intl* primers. The sequence between the 5′ and 3′ conserved region is variable in length and multiple bands have been reported with Class 1 integron primers by other workers for different *Salmonella* serovars[25,26]. Our finding is, therefore, concordant with published papers.

The multiplex PCR analysis was performed using a range of bacteria other than clinical *S.* Typhi isolates *viz.* *S.* Typhimurium ATCC 14028 and *S.* Paratyphi (laboratory reference strain) and *E. coli* O157:H7 without generation of any nonspecific bands in nontarget bacteria. Our method was, therefore, highly specific. We believe that the method optimized in this study will help in rapid diagnosis and selection of treatment options. However, further experiments

need to be carried out in order to test the sensitivity of the developed protocol.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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