A Pentaplex PCR Assay for the Detection and Differentiation of Shigella Species

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The magnitude of shigellosis in developing countries is largely unknown because an affordable detection method is not available. Current laboratory diagnosis of Shigella spp. is laborious and time consuming and has low sensitivity. Hence, in the present study, a molecular-based diagnostic assay which amplifies simultaneously four specific genes to identify invC for Shigella genus, rfc for S. flexneri, wbgZ for S. sonnei, and rfpB for S. dysenteriae, as well as one internal control (ompA) gene, was developed in a single reaction to detect and differentiate Shigella spp. Validation with 120 Shigella strains and 37 non-Shigella strains yielded 100% specificity. The sensitivity of the PCR was 100 pg of genomic DNA, 5.4 × 10^4 CFU/ml, or approximately 120 CFU per reaction mixture of bacteria. The sensitivity of the pentaplex PCR assay was further improved following preincubation of the stool samples in Gram-negative broth. A preliminary study with 30 diarrhoeal specimens resulted in no cross-reaction with other non-Shigella strains tested. We conclude that the developed pentaplex PCR assay is robust and can provide information about the four target genes that are essential for the identification of the Shigella genus and the three Shigella species responsible for the majority of shigellosis cases.

1. Introduction

Shigellosis continues to be a major health problem in many parts of the world, particularly in underdeveloped and developing countries with poor sanitary systems and improper treatment of water supplies, and also among travelers from industrialized nations [1, 2]. Worldwide, mortality and morbidity due to shigellosis were found to be highest among young children 1 to 5 years of age and the elderly [3–5]. Three species of Shigella are responsible for the majority of shigellosis cases: S. flexneri, S. sonnei, and S. dysenteriae. Of these, S. sonnei is encountered mostly in industrialized countries and S. flexneri in developing countries; S. dysenteriae is the only epidemic and pandemic strain [2, 4, 6, 7]. The pathogenesis of shigellosis includes inflammation, ulceration, haemorrhage, tissue destruction, and fibrosis of the colonic mucosa, which result in abdominal pain and diarrhoea/dysentery; in some cases infertility and endometriosis also have been reported [8, 9]. Bacteraemia may occur in people with severe infections, particularly in malnourished children and AIDS patients [10]. A more recent annual estimate of shigellosis throughout the world was estimated to be 90 million incidences and 108,000 deaths [11].

Shigella infection spreads by the faecal-oral route. Because of the low infectious dose (10 to 100 organisms), person-to-person transmission is likely the most common route of infection, as the bacteria can survive gastric acidity better than other enteric bacteria [10, 12]. However, transmission via contaminated water, food, overcrowded communities, food handlers, contaminated swimming pools, and flies also has been documented [8, 13, 14]. Recent increases in the number of cases of shigellosis in many parts of the world are attributed to the emergence of multiple-drug resistant strains. Early and accurate diagnosis of shigellosis coupled with prompt medical intervention is essential for reducing the morbidity and mortality caused by Shigella spp.
Shigella spp. are fragile organisms that are excreted in large numbers in the stool, but they die off quickly because stools are acidic [15]. Thus, routine microbiological methods used to identify Shigella spp. from stool samples are relatively inefficient, time consuming, and labor intensive, and the diagnosis often remains obscure due to the presence of low numbers of causative organisms, competition from other commensal organisms, and inappropriate sample collection. If samples are collected after antibiotic therapy, growth of the organism may be impaired. Moreover, Dutta et al. [16] and Islam et al. [17] reported the sensitivity of the culture method to be 54% and 74%, respectively, compared to that of the conventional PCR technique. Recent molecular diagnostic techniques based on nucleic acids, such as PCR, have shown tremendous potential for identifying Shigella spp. and have been increasingly exploited.

To date, few studies have focused on the rapid diagnosis of shigellosis in underdeveloped and developing countries. However, PCR diagnostic tests have proven to be rapid and effective for the detection and identification to Shigella spp. [16–18]. In this study, we searched for genes unique to the Shigella serovars and used them to design a pentaplex PCR assay. Our assay differs from conventional multiplex PCRs, which often target the invasion plasmid H (ipaH) gene, O antigen synthesis genes, and the 16S rRNA gene for detection of Shigella spp. [18–20]; in those cases, the diagnosis is often based on sequence polymorphisms or differences rather than on the absence or presence of a gene. Those methods do not detect Shigella at the genus and species level simultaneously. The goal of the present study was to design a pentaplex PCR of Shigella spp. with an internal control for the detection of the genus Shigella and also for the clinically important Shigella spp., namely, S. flexneri, S. sonnei, and S. dysenteriae.

2. Methods

2.1. Bacterial Strains and Growth Conditions. A total of 120 Shigella strains of S. flexneri (n = 95), S. sonnei (n = 20), S. dysenteriae (n = 3) and S. boydii (n = 2), were used in this study. Pure culture strains were isolated from patients admitted to Hospital Universiti Sains Malaysia (HUSM) from 2001 to 2009. Table 2 lists the Shigella spp. reference strains and other bacteria used in this study. Non-Shigella strains were used to determine the specificity and robustness of the assay. All the strains were biochemically and serologically confirmed and were stored at −80°C in 16% glycerol.

2.2. Isolation of Shigella Spp. from Clinical Specimens Using a Conventional Method. Stool specimens were inoculated on MacConkey (Oxoid Ltd., UK) and deoxycholate citrate agar (DCA) (Oxoid Ltd., UK) using a sterile inoculating loop. Stools were also enriched in selenite F broth (Oxoid Ltd., UK) and incubated overnight at 37 ± 2°C. The next day, the enriched broth was subcultured on MacConkey agar and DCA and incubated overnight at 37 ± 2°C. Colonies morphologically resembling Shigella spp. were further evaluated with biochemical tests using triple sugar iron (Oxoid Ltd., UK), urea agar slant (Oxoid Ltd., UK), methyl red (Oxoid Ltd., UK), Simmon’s citrate agar slant (Oxoid Ltd., UK), and sulphur indole motility medium (Oxoid Ltd., UK). Identities of colonies were serologically confirmed by slide agglutination with appropriate group-specific polyvalent antisera followed by type-specific monovalent antisera (Denka-Seiken, Tokyo, Japan). Nonserotypable isolates were further checked using an API 20E kit (BioMerieux, Marcy l’Etoile, France).

2.3. Primer Design for Pentaplex PCR Assay. The gene sequence for invC of the genus Shigella and gene sequences for rfc, wbgZ, and rfpB of S. flexneri, S. sonnei, and S. dysenteriae, respectively, were obtained from GenBank [21] for DNA sequence alignment and primer design. The ClustalW program in Vector NTI version 9.0 software (Invitrogen, Carlsbad, CA, USA) was used to align the DNA sequences. The conserved and non-conserved regions of the DNA sequence alignments were visualized using GeneDoc software [22].

Based on the conserved regions of the alignment, specific primer pairs for the genus Shigella were designed to amplify the invC gene. Specific primers for S. flexneri, S. sonnei, and S. dysenteriae were designed based on the non-conserved regions of rfc, wbgZ, and rfpB genes, respectively. The four primer pairs were designed in such a way that amplification efficiency was not hindered and amplicon sizes ranging from 211 to 875 bp could be differentiated by agarose gel electrophoresis (Figure 1). The homology of the designed primer sequences was analyzed using BLAST [21]. A primer pair based on the ompA gene was designed (1319 bp) and used as an internal control. The primer (A1T BIOTECH, Singapore) sequences for the five genes and expected PCR product sizes are shown in Table 1.

2.4. Pentaplex PCR Assay. The pentaplex PCR assay was standardized using genomic DNA extracted from reference Shigella spp. A mixture of DNA from three strains (S. flexneri (SH052), S. sonnei (SH023), and S. dysenteriae (SD375)) that contained the four genes of interest was used as a positive control. DNase-free distilled water was used as a negative control. In addition, a plasmid containing the ompA gene (10 pg) was incorporated as an internal control template to rule out false negative results. An internal control (primer pair and template) was incorporated into every reaction mixture, including negative controls.

The colonies isolated from blood agar were inoculated into nutrient agar (Oxoid Ltd., UK) and incubated overnight at 37 ± 2°C. Bacteria lysate was prepared by resuspending one bacterial colony in 30 μL of deionized water, boiling for 5 min, and centrifuging at 8000 × g for 2 min. Two microliters of supernatant then were used as the DNA template in the pentaplex PCR assays.

The optimized primer concentration for each gene (0.4 pmol for ompA, rfc, and rfpB; 0.3 pmol for invC; and 0.2 pmol for wbgZ) was used in the pentaplex PCR. The other components used in the PCR were 200 μM dNTPs, 2.5 mM MgCl2, 1X PCR buffer, and 1 U Taq DNA polymerase (Promega, Madison, WI, USA). The PCR was performed using a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with one cycle of initial denaturation at 94°C for 3 min,
**Figure 1:** Pentaplex PCR assay profile with reference strains. M, 100 bp plus marker; lane 1, negative control; lane 2, positive control; lane 3, SH052 strain (*rfc* S. flexneri, *invC* Shigella genus); lane 4, SH031 strain (*invC* Shigella genus, *wbgZ* S. sonnei); lane 5, SD375 strain (*invC* Shigella genus, *rfpB* S. dysenteriae); M, 100 bp plus marker.

**Table 1:** Sequences of primers used for the pentaplex PCR.

| Primers        | Primer sequence (5'-3') | Gene target | Location of gene | Amplicon size (bp) | Target identity   | GenBank accession number |
|----------------|-------------------------|-------------|------------------|--------------------|--------------------|--------------------------|
| SgenDF1        | TGC CCA GTT TCT TCA TAC GC | *invC*      | Plasmid          | 875                | Shigella genus     | AF386526                 |
| SgenDR1        | GAA AGT AGC TCC CGA AAT GC |             |                  |                    |                    |                          |
| SflexDF1       | TTT ATG GCT TCT TCG GC   | *rfc*       | Chromosome       | 537                | Shigella flexneri  | CP000266                 |
| SflexDR1       | CTG CGT GAT CCG ACC ATG  |             |                  |                    |                    |                          |
| SsonDF1        | TCT GAA TAT GCC CTC TAC GCT | *wbgZ*     | Plasmid          | 430                | Shigella sonnei    | CP000039                 |
| SsonDR1        | GAC AGA GCC CGA AGA ACC G |             |                  |                    |                    |                          |
| SdysDF1        | TCT CAA TAA TAG GGA ACA CAG C | *rfpB*    | Plasmid          | 211                | Shigella dysenteriae| CP000640                 |
| SdysDR1        | CAT AAA TCA CCA GGA AGG TT |             |                  |                    |                    |                          |
| ICF1           | GCA GGC ATT GCT GGG TAA  | *ompA*      | Plasmid          | 1319               | Internal control   | AY305875                 |
| ICDRI          | ACA CTT GTA AGT TTT CAA CTA C   |             |                  |                    |                    |                          |

30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 30 s, followed by an extra cycle of annealing at 60°C for 30 s and a final extension at 72°C for 3 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels (Promega) with 10 mg/mL ethidium bromide (Sigma, USA); they were run at 100 V for 60 min. PCR products were visualized under a UV transilluminator and photographed using an image analyzer (Chemilager 5500; Alpha Innotech, San Leandro, CA, USA).

**2.5. Evaluation of Pentaplex PCR Assay Results.** Analytical specificity was evaluated using DNA lysate prepared from pure cultures of 120 *Shigella* strains, 10 Gram-positive strains, and 27 Gram negative strains. The analytical sensitivity was evaluated using genomic DNA (1 μg to 10 pg) and also 10⁵ to 10⁷ CFU/mL obtained from *Shigella* strains. The diagnostic evaluation of the pentaplex PCR was conducted using 95 *S. flexneri*, 20 *S. sonnei*, 3 *S. dysenteriae*, and 2 *S. boydii* strains. The results were compared with those from the conventional culture method, which is considered to be the standard of detection [23].

**2.6. Faecal Spiking and Sensitivity.** The standardized pentaplex PCR assay designed to detect *Shigella* directly from stool was also tested using stool samples spiked with a known amount of *Shigella* based on slight modification of method described by Houng et al. [18]. Stool samples (*n* = 2, children ≤ 5 years old) were collected from the Department of Medical Microbiology and Parasitology, HUSM, Malaysia, and were pretested for the presence of amplifiable *Shigella* DNA by pentaplex PCR and found to be negative. Five grams of stool were weighed and suspended in 45 mL of normal saline (NS) solution, which corresponds to a 10% mixture. The solution was vortexed for 2 min to obtain a homogenous mixture. Insoluble particulate matter was removed by low-speed centrifugation (1000 xg) for 3 min, and the supernatant was transferred to a fresh tube. Meanwhile, an overnight culture of *Shigella*-specific strains was grown in nutrient broth (NB)
The optimum concentration of primer needed to amplify the assay and to exclude false negative results. Figure 1 shows a representative gel that illustrates differentiation of pentaplex PCR but negative for non- Shigella spp. [28], seminested PCR [29], PCR-radioactive labeling [30], PCR-RFLP [31], and PCR-ELISA [32]). On the other hand, DNA microarray analysis proved to be specific, sensitive, and reproducible, but its application as a diagnostic or epidemiological tool is difficult in view of the elevated cost, instruments and requires a skilled person to perform the test [33].
To overcome these drawbacks of existing techniques, we developed a pentaplex PCR assay and evaluated its ability to detect and identify three enteropathogenic bacteria species at the genus and species levels. Several previous studies described the development of *Shigella* multiplex PCR, but those assays did not discriminate between *Shigella* at the genus and species levels, nor did they differentiate *Shigella* from closely related pathogens such as *Salmonella*, *Citrobacter*, and enteroinvasive *Escherichia coli* (EIEC) [20, 25, 34].

| Bacterial strains | No. of strains tested | invC | rfc | wbgZ | rfpB | IC (ompA) |
|-------------------|-----------------------|------|-----|------|------|----------|
| *S. flexneri* (ATCC 12022)\(^b\) | 1 | +  | +  | −    | −    | +        |
| *S. sonnei* (SH031)\(^c\) | 1 | +  | −  | +    | −    |          |
| *S. boydii* (ATCC 9207)\(^b\) | 1 | +  | −  | −    | −    | +        |
| *S. dysenteriae* (SD375)\(^d\) | 1 | +  | −  | −    | +    | +        |
| *Salmonella* spp. | 2 | −  | −  | −    | −    | +        |
| *S. Typhi* \(^c\) | 3 | −  | −  | −    | −    | +        |
| *S. Paratyphi A* \(^c\) | 1 | −  | −  | −    | +    |          |
| *S. Paratyphi B* \(^c\) | 1 | −  | −  | −    | +    |          |
| *Klebsiella* spp.\(^c\) | 2 | −  | −  | −    | −    | +        |
| *K. pneumoniae* \(^c\) | 2 | −  | −  | −    | −    | +        |
| *E. coli* (EPEC)\(^c\) | 1 | −  | −  | −    | −    | +        |
| *E. coli* (EHEC)\(^c\) | 1 | −  | −  | −    | −    | +        |
| *E. coli* (ETEC)\(^c\) | 1 | −  | −  | −    | −    | +        |
| *E. coli* \(^c\) | 4 | −  | −  | −    | −    | +        |
| *V. cholerae* \(^c\) | 3 | −  | −  | −    | −    | +        |
| *V. parahemolyticus* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *V. fulvalis* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *V. cholera* (wild type)\(^c\) | 1 | −  | −  | −    | −    | +        |
| *V. furnissi* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *P. aeruginosa* \(^c\) | 3 | −  | −  | −    | −    | +        |
| *P. mirabilis* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *P. vulgaris* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *C. fraudii* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *E. cloacae* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *Y. enterocolitica* \(^c\) | 1 | −  | −  | −    | −    | +        |
| Acinetobacter spp. \(^c\) | 1 | −  | −  | −    | −    | +        |
| *A. baumannii* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *S. marcescens* \(^c\) | 1 | −  | −  | −    | −    | +        |
| Campylobacter spp. \(^c\) | 1 | −  | −  | −    | −    | +        |
| *A. hydrophila* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *M. morganii* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *B. cereus* \(^c\) | 1 | −  | −  | −    | −    | +        |
| *S. aureus* \(^c\) | 2 | −  | −  | −    | −    | +        |
| Methylene resistant *S. aureus* \(^c\) | 1 | −  | −  | −    | −    | +        |
| Streptococcus spp. Group A \(^c\) | 1 | −  | −  | −    | −    | +        |
| Streptococcus spp. Group B \(^c\) | 1 | −  | −  | −    | −    | +        |
| Streptococcus spp. Group G \(^c\) | 1 | −  | −  | −    | −    | +        |
| Corynebacterium spp. \(^c\) | 1 | −  | −  | −    | −    | +        |
| Listeria spp. \(^c\) | 1 | −  | −  | −    | −    | +        |
| Lactobacillus spp. \(^c\) | 1 | −  | −  | −    | −    | +        |
| Gardnerella spp. \(^c\) | 1 | −  | −  | −    | −    | +        |

\(^a\)Shigella genus.

\(^b\)Reference strains from American Type Culture Collection (ATCC), Reston, VA, USA.

\(^c\)Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia.

\(^d\)Obtained from Institute for Medical Research, Malaysia.

"+" is positive; "−" is negative by pentaplex PCR.
In our study, primers were designed based on the prevalent species responsible for the majority of shigellosis cases [2, 4, 6, 7]. Four highly specific genes (invC, rfc, wbgZ, and rfpB) that can best detect Shigella at the genus and species level were identified. Because invC is present among all of the Shigella spp., rfc, wbgZ, and rfpB were combined with invC for speciation of the Shigella strains. The primer for S. flexneri that targets the rfc gene was designed based on Houngetal. [18], and it allows discrimination between Shigella and EIEC in faecal samples. Similarly, the three other highly specific primers were designed based on the homologous sequences retrieved from GenBank (NCBI). S. boydii species identification was not included in this study because of its low prevalence in developing and industrialized countries. However, the presence of the invC band specific for Shigella genus and the absence of all other amplicons specific for Shigella spp. can be considered to be the detection criteria for S. boydii.
Following the successful application of the primers individually, they were mixed to produce the pentaplex PCR. The mixing of primers in a single tube decreases costs and time and increases the ease of the assay. Although numerous reports of PCR assays for the detection of *Shigella* spp. exist [18, 20, 25, 34], only a few of them have incorporated internal controls to rule out false negatives [35]. According to guidelines for Molecular Diagnostic Methods for Infectious Diseases (MM3-A2), incorporation of an internal control in the reaction is essential for the diagnostic test to exclude false negative result or the presence of inhibitors. In the present study, inclusion of a 1319 bp internal control in the pentaplex PCR assay helped us to rule out false negatives or PCR inhibitors. The primers were designed with great care; BLAST and alignment results of the sequence confirmed that it did not cross-react with closely related species such as enteroinvasive *Escherichia coli* (EIEC) which gives rise to similar illness as shigellosis. However, it was unfortunate that EIEC strain was not available to be tested in this study.

The pentaplex PCR developed in our study successfully amplified all five amplicons from a single reaction tube, and the primers did not interact with each other to produce false negatives. Compatibility of primers with target amplicons was confirmed by sequencing the PCR products derived from the five representative strains. The pentaplex mixture was tested with 120 clinical strains and also against other Gram positive organisms, including some coliform bacteria. The amplification and limit the usefulness of PCR technique [36, 37]. As reported by Theron et al. [29] and Thong et al. [20], an enrichment procedure prior to PCR enhances the total number of bacteria present, which helps to dilute the PCR inhibitory substances. As stated by the manufacturer of Gram negative broth (GNB), citrate and deoxycholate in the broth act as selective agents and suppress the growth of Gram positive organisms, including some coliform bacteria. The additional step of preincubating spiked faecal sample in GNB helps to eliminate the natural inhibitors and could enhance the viability of *Shigella* spp. in samples [29, 38]. A preliminary study with clinical specimens showed no cross reaction with other non-*Shigella* strains, however, to check the real performance of the developed test, a larger positive sample size need to be further investigated. The 4 h enrichment step would increase the total number of bacteria present and enhance the sensitivity of the assay. The sensitivity level achieved in our study was comparable to that of other studies. For example, Houng et al. [18] detected up to $7.4 \times 10^4$ CFU/mL of *Shigella* by amplifying IS 630 sequences, Yavzori et al. [39] detected at $10^4$ CFU of *Shigella* per gram of faeces with the use of virF primers, and Thong et al. [20] reported a detection level of $5.0 \times 10^4$ CFU/mL of *Shigella* by amplifying ial and ipaH sequences in *Shigella* spp. Thus, the average detection of pentaplex PCR described in this study ($5.4 \times 10^4$ CFU/mL) is within the common detection limit for *Shigella*.

### 5. Conclusion

In conclusion, the pentaplex PCR assay developed in this study was able to detect four genes that are essential for the detection and differentiation of *Shigella* at the genus and species levels simultaneously in a single test within 4 h. The built-in internal control in this assay prevented false negative results. The pentaplex PCR assay was highly sensitive and could provide results on the same day that a specimen was submitted for evaluation, which is critical during outbreaks.

### Conflict of Interests

The authors declare that they have no conflict of interests.

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