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SHORT REPORT

Detection of *Shigellae* from Stools of Dysentery Patients by Culture and Polymerase Chain Reaction Techniques

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Abstract

In Bangladesh, the isolation rates of *Shigella* spp. range from 11% to 12% by the conventional culture technique. Since the sensitivity of this technique is low, the polymerase chain reaction (PCR) technique was used for detecting small number of *Shigellae* from patients' stools. Sensitivity and specificity of the two techniques were also compared. Stool samples were collected from 41 patients with dysentery who attended the Clinical Research and Service Centre of the ICDDR,B: Centre for Health and Population Research. All stool specimens were directly plated onto MacConkey, Salmonella-Shigella, Xylose lysin deoxycholate and Hectoen enteric agar media, and *Shigellae* were detected following standard procedures. DNA was extracted from the stool samples, and the target sequence of invasive plasmid antigen *(ipa)*H locus was amplified by PCR with 130 ng each of two primers (primer H8 [5'-GTTCCTTGACCGCCTTTCCGATAC-3'] and primer H15 [5'-GCCGGTCAGCCACCCTA-3']) following standard procedures. The amplified product was hybridized using an *ipa*H probe. The isolation rates of *Shigella dysenteriae* type 1, *S. flexneri, S. sonnei,* and *S. boydii* were, respectively, 17.1%, 19.5%, 4.9% and 2.4% by the conventional method. The results of the PCR technique showed that 700 bp fragment was generated in 18 of the 18 culture-positive and in 7 of the 23 culture-negative stools. One hundred twenty-three strains of *Escherichia coli* were also tested by PCR for identifying the enteroinvasive *E. coli,* but none of them yielded any positive result. This study showed that the sensitivity of the culture technique is 72% and specificity is 100%, when the PCR technique was considered as gold standard. Therefore, the PCR may be considered a more sensitive and specific technique than the conventional culture technique and has the potential to be employed in routine diagnosis of dysentery in clinical centres as well as in epidemiologic studies.

Key words: *Shigella*; Dysentery, Bacillary; Culture media; Polymerase chain reaction; Comparative studies

INTRODUCTION

Shigella species are the recognized pathogens of bacillary dysentery and have an isolation rate of approximately 11-12% in diarrhoea patients in Bangladesh, using the conventional culture technique (4). But the performance of the culture technique is limited by its low sensitivity. As a result, shigellosis remains undiagnosed in a significant number of patients (1). Besides, during the convalescent period, *Shigellae* may remain undetected in stools when examined by the conventional culture

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technique due to the presence of fewer organisms as well as non-culturable forms of the organism (5).

The polymerase chain reaction (PCR) technique, on the other, seems ideally suited to detect low number of *Shigellae,* since this method enables the amplification of DNA obtained from as few as a single cell present in the sample (10). This method depends only on the presence of target DNA and allows a specific segment of DNA to be amplified by a factor of 10^6 or more within hours (8). The PCR technique was used in two studies for detecting *Shigellae* from stools (1,2). An invasive-associated locus (*ial*) probe was used in these studies. The *ial* sequence is present in plasmids, as the plasmid is unstable, it can be easily lost (6). To overcome this problem, the invasive plasmid antigen H (*ipa*H) locus probe can be used, since this gene is present in more than one copy on the invasive plasmid and chromosome (3). Although the PCR technique is superior, adequate laboratory trials are required for its optimal application. We, therefore, used both culture and PCR techniques with *ipa*H probe to assess their performance in detecting *Shigellae* from stools of dysentery patients.

MATERIALS AND METHODS

Stool samples, collected from 41 patients with dysentery who attended the Clinical Research and Service Centre of the ICDDR,B: Centre for Health and Population Research, were directly plated onto MacConkey, Salmonella-Shigella, Xylose lysin deoxycholate and Hektoen enteric agar plates (Difco, Detroit, Michigan, USA) to detect *Shigellae* following procedures described by Islam *et al*. (5). In brief, the inoculated plates were incubated at 37 ^{o}C for 18-24 hours. Suspected *Shigella*-like colonies from the plates were subsequently inoculated onto Kligler iron agar, motility indole urea and Simmons citrate agar media. Non-motile organisms showing acid butt and alkaline slant and lack of gas and H_2S production in Kligler iron agar, and negative reaction for urea and citrate utilization were tentatively identified as *Shigellae*. They were then confirmed by slide agglutination with specific *Shigella* antiserum (Wellcome Diagnostics, Dartford, England). Since the *ipa*H genes are also present in enteroinvasive *Escherichia coli,* 123 *E. coli* strains (3 strains each from 41 stool samples) from MacConkey plates were tested by the PCR technique.

One hundred µL of stool was mixed in 0.5 mL of phosphate-buffered saline in a 1.5-mL microfuge tube. Suspending particles were removed by centrifugation at 503 g for 3 minutes. The supernatant was again centrifuged at 11,000 rpm for 3 minutes. DNA was extracted from the pellet following procedures described by Echeverria *et al*. (1), and the target sequence of *ipa*H locus was amplified by PCR with 130 ng each of two primers (primer H8 [5'-GTTCCTTGACCGCCTTTCCGATAC-3'] and primer H15 [5'-GCCGGTCAGCCACCCTA-3']) (3) and 1.25 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) per 25 µL of reactions mixture on a Thermal Cycler (Perkin-Elmer Cetus). The PCR reaction was done for 35 cycles of 1 minute each at 94 ^{o}C (for denaturation), 1.5 minute each at 60 ^{o}C (for annealing of primers to single-stranded DNA), and 0.25 to 1 minute each at 72 ^{o}C (for DNA polymerase-mediated extension). The amplified products were then separated by agarose (0.8%) gel electrophoresis, transferred to a nylon membrane by southern blotting and subjected to hybridization with the *ipa*H probe (5). The probe is basically the fragment amplified with primers H8 and H15. Labelling and detection of the hybridized probe were performed with an enhanced chemiluminescence gene detection system following the instructions of the manufacturer (Amersham International, UK). The validity of the primers was previously tested, the results of which indicated that these primers were highly sensitive and specific for detection of *Shigellae* (5,9).

RESULTS

Shigellae were isolated from 18 (43.9%) of the 41 dysentery patients using the culture technique, and the isolation rates of *S. dysenteriae* type 1, *S. flexneri*, *S. sonnei*, and *S. boydii* were 17.1%, 19.5%, 4.9% and 2.4% respectively.

The extracted DNA of the faecal samples was specifically subjected to amplification for *ipa*H sequence using *Shigella*-specific (*ipa*H) primers in Thermal Cycler; it was found that after 35 cycles of amplification, 700 bp fragment was generated in 18 of the 18 culture-positive and in 7 of the 23 culture-negative stool samples (Fig.1A). This amplified fragment was confirmed by hybridization with *ipa*H probe (Fig. 1B). Therefore, *Shigellae* were detected in 25 (61.0%) of the 41 stool samples, using the PCR technique. But none of the 123 *E. coli* strains yielded any positive result with PCR.

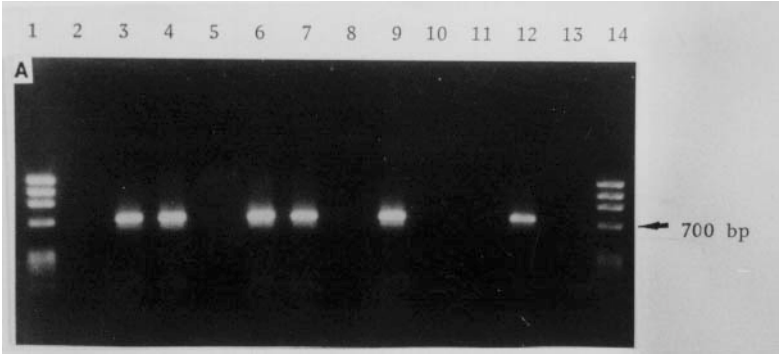

Fig 1A: Specific amplification of invasion plasmid antigen H (*ipa*H) locus DNA by PCR. Amplified products of each reaction were analyzed by electrophoresis through a 0.8% agarose gel. Lanes: 1 and 14, *Hae* III digest of øX 174 replicative form DNA; 2 and 13, negative controls without template DNA; 3, *S. dysenteriae* 1 used as the positive control; 4, 6, 7 and 9, *S. flexneri, S. sonnei, S. boydii,* and *S. dysenteriae* isolated from stools respectively; 5, 8, 10 and 11, PCR negative and no *Shigella* species isolated; 12, PCR positive, but no *Shigella* species isolated.

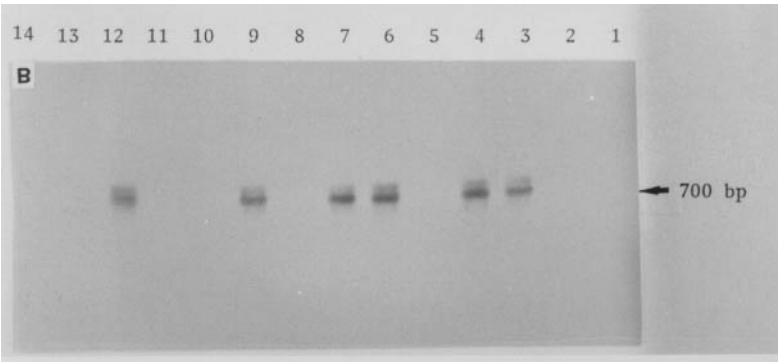

Fig 1B: Hybridization of southern blot shown in panel A with *ipa*H DNA probe as detected by the enhanced chemiluminescence technique. Each signal corresponds to the amplified PCR product.

DISCUSSION

The PCR technique can detect a small number of culturable as well as non-culturable organisms. Such detection is specially important for *Shigellae*, since they can produce disease by as few as 10-100 organisms (7). In our study, the use of PCR technique improved the rate of detecting *Shigellae* in stool samples from 43.9% to 61.0%; which is 17% higher than the conventional culture method. Moreover, the time requirement of this technique is lower compared to that of the culture technique. The detection scheme, including the DNA extraction, DNA amplification and analysis of amplified products, requires only 5-6 hours compared to 18-24 hours of standard stool culture. The specificity of the culture technique is 100%, and the sensitivity is 72%, when the PCR technique is considered as gold standard. Thus, PCR may be judged as superior for its rapidity and sensitivity in the detection of *Shigellae*. Moreover, since this method is applied without employing cultivation of organisms on the synthetic medium, non-culturable population of *Shigellae* can also by detected by this method.

In view of the above, PCR may, therefore, be considered a more sensitive and specific technique than the conventional culture technique, and has the potential in routine diagnosis of dysentery in clinical centres as well as in epidemiologic studies. The PCR technique can be chosen as an alternative to the culture technique, and can further be used for identifying asymptomatic carriers, serving as potential reservoirs of *Shigellae* silently transmitting the disease within communities. Based on the findings of our study, it can be concluded that the PCR technique is more useful than the conventional culture technique in precisely determining the actual prevalence rate of *Shigellae* among dysenteric patients.

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