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Rapid PCR Detection of *Salmonella* from Stool Samples

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ABSTRACT

Salmonellosis is one of the leading causes of acute enteric infection in humans and animals. The present study was undertaken to develop a Polymerase Chain Reaction (PCR) protocol for rapid detection of *Salmonella* directly from the stool samples. A PCR was optimized targeting *inv*A gene of *Salmonella*. The sensitivity of the PCR was found to be $8X10^2$ cfu/ml from serially diluted bacterial culture, whereas the sensitivity in spiked stool sample after 4 h enrichment was found up to $2X10^2$ cfu/ml. The study showed that PCR is highly sensitive and specific diagnostic tool for rapid and specific detection of *Salmonella* from stool samples. This can be used for epidemiological investigations, where immediate diagnosis is needed for patient management.

Keywords: *inv*A, PCR, *Salmonella*, stool

Introduction

Salmonellosis has become one of main causative agents of enteric infections in human and animal with more than 2541 serotypes responsible for large number of infections (Anon, 2007; Kumar *et al*., 2012). The gold standard diagnostic method for salmonellosis is culture of the bacteria from the samples, which is a time-consuming and tedious process and may involve 4 to 7 days to identify and confirm *Salmonella* from samples (Van der Zee and Huis in't Veld, 2000). The organism may not be detected in certain clinical samples when present in small numbers (Fricker, 1987) and sometime very small numbers of viable organisms present in the faecal clinical sample may fail to grow in artificial media. Besides, there are some isolates that maintain atypical culture characteristics (lack of H_2S production, lactose fermentation, etc) leading to misdiagnosis by bacterial culture. Several techniques for improving the detection of *Salmonella* in faeces, such as the use of a selective culture medium and enzyme-linked immunosorbent assay have been developed. However, problem remains with sensitivity and specificity that have limited the routine use of these diagnostic methods. Therefore, development of a rapid and sensitive method for the diagnosis of *Salmonella* is desirable. Molecular techniques have been found to be most successful in areas for which conventional microbiological techniques do not exist, or are too slow or are too expensive (Jungkind, 2001). Molecular tools like PCR allow rapid identification of organism in 3 to 24 h. It is the best known and most successfully implemented nucleic acid detection technology (Nissen and Sloots, 2002). This may also be useful in epidemiologic investigations, where immediate identification of infected individuals is needed to make patient management decisions.

The *inv*A gene of *Salmonella* is conserved virulent gene and has been proved to be a suitable PCR target with potential diagnostic application for detection of *Salmonella*. It is also recognized as an international standard for detection of *Salmonella* genus (Malorny *et al*., 2003; Jamshidi *et al*., 2008). Therefore, the present study targeted PCR amplification of *inv*A gene of *Salmonella* for its direct detection from stool sample.

Materials and Methods

DNA amplification and detection

Optimization of PCR targeting *inv*A gene of *Salmonella* was done using a strain of *Salmonella* Typhimurium. The same strain was used for spiking of stool sample to know the sensitivity of PCR. A 26-bp forward primer (5′- GTG AAA TTA TCG CCG CGT TCG GGC AA3′) and a 22-bp reverse primer (5′ TCA TCG CAC CGT CAA AGG AAC C 3′) (Rahn *et al*., 1992) targeting the *inv*A gene of *S.* Typhimurium, were used in PCR to obtain a 284 bp product. Amplification was carried out in a total volume of 25 µl containing 10 pmol each primer, 50 µM each

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dNTP, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase, 1X PCR buffer and 5 µl template DNA. Template DNA was prepared by boiling and snap chilling method. A negative control containing the same reaction mixture except the DNA template was included in every experiment. An initial denaturation at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min. Finally, an additional extension was achieved for 5 min at 72°C. The PCR product was electrophoresed on a 1·5% agarose gel at 100 V, stained in ethidium bromide (0·5 µg ml^{-1}) and visualized and photographed under gel documentation system (Biorad).

Sensitivity of PCR

Bacterial cell dilutions

An overnight culture of *S.* Typhimurium was serially diluted (10-fold) with nutrient broth. A 100 µl aliquot of each dilution was boiled for 10 min, snap-cooled and then centrifuged for 1 min at 10000 rpm. A 5 µl aliquot of the supernatant was used as template in the PCR. Viable counts were obtained by plating 100 µl of each dilution of bacterial culture on Nutrient agar plates and incubating overnight at 37°C (Miles and Misra, 1938).

Spiked stool samples

Spiking of stool samples with different concentrations of *S.* Typhimurium was performed as per the method described by Pathmanathan *et al*. (2003). A stool specimen from a healthy individual was diluted 10-fold with NSS and the mixture was spiked with serial 10-fold dilutions of *S*. Typhimurium culture by adding 250 µl of each diluted culture into 250 µl NSS/faeces mixture and the spiked mixture was incubated at 37°C for 0-4 h. The inoculated samples were sampled after 0 and 4 h and PCR was performed using boiled suspensions from the different time-points. The sensitivity of the PCR was defined as the lowest concentration of *S*. Typhimurium (in c.f.u. ml^{-1} or per PCR) that yielded positive results. To rule out false positivity, a negative control containing un-spiked stool suspension was included in every experiment.

Specificity of PCR

Specificity of the PCR was determined by using DNA template from organism other than *Salmonella* in the reaction mixture.

Results and Discussion

The PCR assay developed in this study was based on amplification of *inv*A gene fragment of *Salmonella*. The target gene was amplified and yielded 284 bp product size (Fig. 1). The non *Salmonella* strain did not produce amplification product under these reaction conditions. The detection limit of the PCR targeting *inv*A gene was determined and found to be $8X10^2$ cfu/ml in serial dilutions of bacterial cell culture (Fig. 2). This was equivalent to 16 cfu per PCR reaction. The sensitivity of the PCR in spiked stool sample was determined and found to be $2X10^6$ cfu/ml when spiked stool samples were directly (without enrichment) used as template in PCR (Fig. 3). However, after 4 h enrichment at 37°C, the sensitivity was increased to $2X10^2$ cfu/ml i.e. 2 cfu per PCR (Fig. 4).

Although bacterial culture remains the gold standard for *Salmonella* detection, it is a cumbersome and time taking procedure. This may involve 4 to 7 days to identify and confirm *Salmonella* from clinical samples (Stone *et al*., 1994). Therefore, molecular techniques such as PCR allow rapid identification of *Salmonella* in 3 to 4 h (Amavisit *et al*., 2001).

Entry of *Salmonella* in to the host epithelial cells is guided by invasive gene, *inv*A, located on the pathogenicity island 1 of *Salmonella* species (SPI 1) (Mills *et al*., 1995; Collazo and Galan, 1997). This gene is present in all invasive strains of *Salmonella* and absent in closely related genera such as *Escherichia* (Galan, 1996; Baumler *et al*., 1998). Therefore, in the present study, the specificity and sensitivity of a pair of primers targeting the *inv*A gene of *Salmonella* was assessed for the detection of *Salmonella* species in human faeces. Since the faecal samples contain different PCR inhibitory substances like bilirubin, bile salts and other organic and inorganic material (Stone *et al*., 1994; Chiu and Ou, 1996), it is difficult to detect the organism directly by PCR or the sensitivity of the test will be very poor. This can be eliminated or minimized by DNA extraction from the faecal samples with DNA extraction kit, which is very expensive, or by enrichment of the faecal samples in a suitable broth prior to PCR (Dutta *et al*., 2001). However, some laboratories have described enrichment of faecal samples for 6 h before PCR based detection of organism (Stone *et al*., 1994; Gentry-Weeks *et al*., 2002; Pathmanathan *et al*., 2003). Therefore, in the present study, in order to reduce the PCR inhibitors in faecal samples the samples were diluted 10- to 20-fold in nutrient broth, which is less expensive and easy to prepare. The faecal samples were spiked with different dilutions of *Salmonella* (Pathmanathan *et al*., 2003). The spiked stool samples were incubated at 37°C for 4 h for enrichment prior to PCR in order to eliminate inhibitors, which increased the sensitivity of the PCR assay ($2X\ 10^2$ cfu/ml). However, at 0 h enrichment of sample the sensitivity of PCR decreased to $2X\ 10^6$ cfu/ml. This support the enrichment of diluted faecal samples for 4 h resulting into 10^4 time increase in the sensitivity of PCR. The result is in accordance with the finding of Soria *et al*. (2012). The results were obtained in less than 9 h which

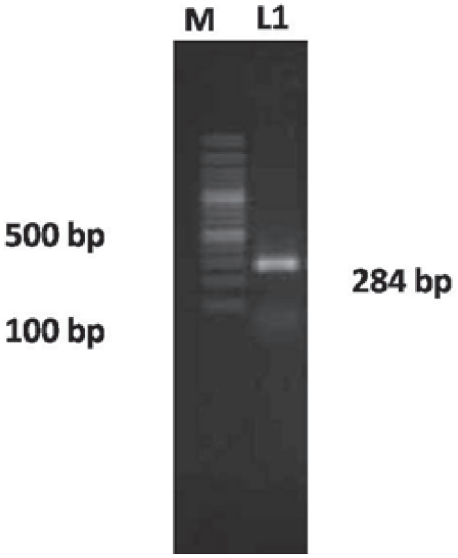

Fig. 1. Optimization of PCR targeting *invA* gene of S. Typhimurium strain.
M - 100 bp plus DNA ladder, L1 - Amplification of *invA* gene of 284 bp size

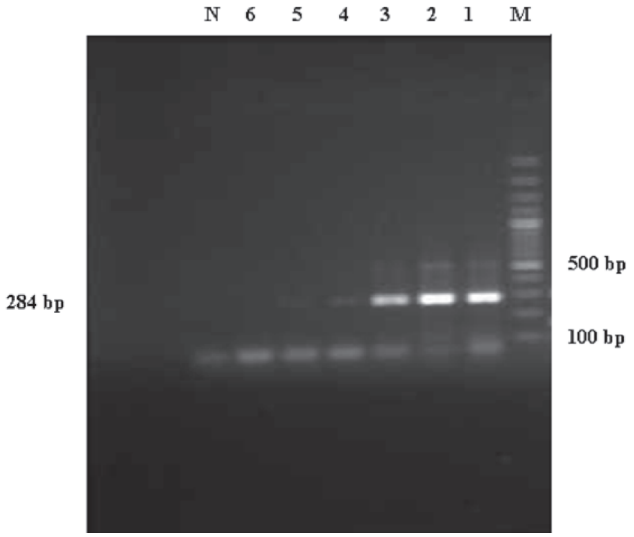

Fig. 2. Sensitivity of the PCR for detection of S. Typhimurium E2375 strtain.
M - 100 bp plus DNA ladder, L1 to L6 - Bacterial culture serially diluted 10-fold from $8X10^6$ (L1) to $8X\ 10^1$ (L6) cfu/ml, N - Negative control.

proved it to be rapid, specific and highly sensitive method. Although, presence of nonspecific bands was observed in PCR, this did not obscure the distinct band of the expected size.

Our results confirmed that *inv*A gene-targeting primers are specific for *Salmonella* and the PCR assay developed in the present study could be a promising technique for diagnosing infections with *Salmonella* using faecal/clinical samples as well as for detecting carriers of *Salmonella* species.

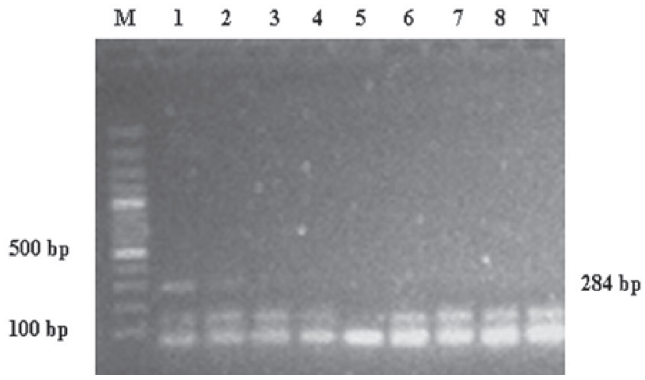

Fig. 3. Sensitivity of PCR for detection of S. Typhimurium in spiked faecal samples without enrichment.
M - 100 bp DNA ladder, L1 to L8 - Faecal samples spiked with bacterial culture serially diluted 10-fold from $8\ X\ 10^7$ (L1) to 8 (L8) cfu/ml, N - Negative control

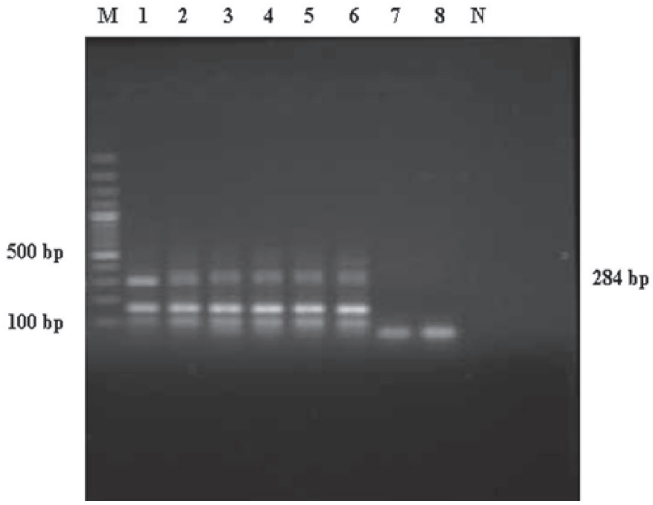

Fig. 4. Sensitivity of the PCR for detection of S. Typhimurium in faecal samples with 4 h enrichment.
M - 100 bp DNA ladder, L1 to L8 - Faecal samples spiked with bacterial culture serially diluted 10-fold from $8\ X\ 10^7$ (L1) to 8 (L8) cfu/ml, N - Negative control.

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