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Simple and rapid detection of *Salmonella* strains by direct PCR amplification of the *hilA* gene

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The suitability of a PCR procedure using a pair of primers targeting the *hilA* gene was evaluated as a means of detecting *Salmonella* species. A total of 33 *Salmonella* strains from 27 serovars and 15 non-*Salmonella* strains from eight different genera were included. PCR with all the *Salmonella* strains produced a 784 bp DNA fragment that was absent from all the non-*Salmonella* strains tested. The detection limit of the PCR was 100 pg with genomic DNA and 3×10^4 c.f.u. ml^{-1} with serial dilutions of bacterial culture. An enrichment-PCR method was further developed to test the sensitivity of the *hilA* primers for the detection of *Salmonella* in faecal samples spiked with different concentrations of *Salmonella choleraesuis* subsp. *choleraesuis* serovar Typhimurium. The method described allowed the detection of *Salmonella* Typhimurium in faecal samples at a concentration of 3×10^2 c.f.u. ml^{-1}. In conclusion, the *hilA* primers are specific for *Salmonella* species and the PCR method presented may be suitable for the detection of *Salmonella* in faeces.

INTRODUCTION

Salmonellosis is responsible for large numbers of infections in both humans and animals (Keusch, 2002). *Salmonella* strains are not detectable in certain clinical samples that contain small numbers of organisms (Fricker, 1987). However, the number of salmonellae present in the faeces of an infected individual is large, i.e. approx. 10^9 g^{-1}. This level of excretion is maintained for several weeks, before falling gradually until the individual no longer excretes (Taylor & McCoy, 1969). Furthermore, after the disappearance of the organism from the intestinal tract, up to 5 % of patients, upon recovery from this disease, may become carriers who shed the organism in their faeces (Jay, 2000). Therefore, detection of *Salmonella* strains in faecal samples is not only important for the diagnosis of salmonellosis, but also essential to identify carriers of this organism, especially among food handlers, who have higher risks of spreading the pathogen.

Conventional methods of isolation of *Salmonella* strains take 4–7 days to complete and are therefore laborious and require substantial manpower (Van der Zee & Huis in't Veld, 2000). Besides, very small numbers of viable organisms present in the faeces may fail to grow in artificial laboratory media. Molecular testing has been most successful in areas for which conventional microbiological techniques do not exist, are too slow or are too expensive (Jungkind, 2001). PCR is the best known and most successfully implemented nucleic acid detection technology to date (Nissen & Sloots, 2002).

Several PCR assays have been developed for the detection of *Salmonella* strains in faeces (Chiu & Ou, 1996; Gentry-Weeks *et al.*, 2002; Stone *et al.*, 1994; Widjojoatmodjo *et al.*, 1992). However, the methods described are either too laborious and time-consuming, too expensive or not sufficiently sensitive. As *Salmonella* infection is the leading food-borne disease in Malaysia (Yasin *et al.*, 1995), we were interested to test a variety of primers to improve target specificity. In the present study, we have evaluated the specificity of a pair of primers reported by Cardona-Castro *et al.* (2002) that targets the *hilA* gene of *Salmonella* strains. The method presented here is rapid, simple, specific and sensitive, as *Salmonella* serovars can be detected directly from faecal samples with no prior extraction of genomic DNA.

METHODS

Bacterial strains. A total of 33 *Salmonella* strains representing 27 different serovars and 15 non-*Salmonella* strains belonging to eight different genera were included in this study. Most strains were provided by the Institute for Medical Research and the University Malaya Medical Centre, Malaysia. They included one strain each of *Listeria monocytogenes* (ATCC 7644^T), *Pseudomonas aeruginosa* (ATCC 27853^T), *Citrobacter freundii*, *Citrobacter koseri*, *Klebsiella pneumoniae*, *Shigella*

flexneri, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella boydii*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Enterobacter cloacae*, *Enterobacter gergoviae*, *Escherichia coli* (ATCC 35421), *Salmonella choleraesuis* subsp. *choleraesuis* serovar Matopeni (*Salmonella* Matopeni), *Salmonella* Stanley, *Salmonella* Thompson, *Salmonella* Corvallis, *Salmonella* Haifa, *Salmonella* Bovismorbificans, *Salmonella* Dublin, *Salmonella* Raus, *Salmonella* Kentucky, *Salmonella* Waycross, *Salmonella* Weltevreden, *Salmonella* Enteritidis, *Salmonella* Muenchen, *Salmonella* Chingola, *Salmonella* Newport, *Salmonella* Virchow, *Salmonella* Hadar, *Salmonella* Infantis, *Salmonella* Lomita, *Salmonella* Hvittingfos, *Salmonella* Blockley and *Salmonella* Bareilly, two strains each of *Salmonella* Typhimurium (lab strain and ATCC 13311^T), *Salmonella* Paratyphi A (lab strain and ATCC 9281), *Salmonella* Paratyphi B (lab strain and ATCC 8759) and *Salmonella* Paratyphi C (lab strain and ATCC 9068) and three strains of *Salmonella* Typhi. *Salmonella* Typhimurium ATCC 13311^T was used to spike stool samples.

PCR primers, DNA amplification and detection. A 30-bp forward primer (5′-CGGAACGTTATTTGCGCCATGCTGAGGTAG-3′) and a 27-bp reverse primer (5′-GCATGGATCCCCGCCGGCGAGATTGTG-3′) (Cardona-Castro *et al.*, 2002), targeting the *hilA* gene of *Salmonella* Typhimurium, were used in PCR to obtain a 784-bp product. Amplification was carried out in a total volume of 50 μl containing 25 pmol each primer, 50 μM each dNTP, 3 mM $MgCl_2$, 1·5 U *Taq* DNA polymerase, 1× PCR buffer and 4 μl template. 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 65 °C for 1 min and extension at 72 °C for 1 min. Finally, an additional extension was achieved for 10 min at 72 °C. A 10 μl aliquot of each PCR product was electrophoresed on a 1·5 % agarose gel for 1·5 h at 100 V, stained for 10 min in ethidium bromide (0·5 μg ml^{-1}) and visualized and photographed under UV illumination.

Specificity of the PCR. All 33 bacterial strains were used to assess the specificity of the PCR. The boiling method was used to prepare the DNA template. A single bacterial colony was picked from the Luria–Bertani (LB) agar plate, boiled in 50 μl distilled water for 10 min and immediately cooled on ice for 5 min. After a short spin, 4 μl of this solution was used in PCR.

Sensitivity of PCR

(i) Genomic DNA. Genomic DNA from *Salmonella* Typhimurium ATCC 13311^T was prepared by a modified method of Saito & Miura (1963). Briefly, 5 ml of an overnight culture grown in LB broth was harvested by centrifugation. The pellet was resuspended in lysozyme solution (1 mg lysozyme ml^{-1} in 0·15 M NaCl, 0·1 M EDTA, pH 8·0), followed by lysis using 1 % SDS, 0·1 M NaCl, 0·1 M Tris/HCl (pH 8·0) at 60 °C. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) in the presence of 5 M sodium perchlorate. A 1/10 volume of 3 M sodium acetate and 2 vols absolute ethanol were added and the nucleic acid was then pelleted by centrifugation, washed with 70 % ethanol and dried under vacuum. The DNA pellet was resuspended in TE buffer (10 mM Tris/HCl, pH 7·5, 0·1 mM EDTA) and then serially diluted with deionized water to concentrations ranging from 100 ng to 1 fg and subjected to PCR amplification.

(ii) Bacterial cell dilutions. An overnight culture of *Salmonella* Typhimurium ATCC 13311^T was serially diluted 10-fold with brain heart infusion (BHI) broth. A 100 μl aliquot of each dilution was boiled for 10 min, snap-cooled and then centrifuged for 1 min at 13 000 r.p.m. A 4 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 LB plates and incubating overnight at 37 °C.

(iii) Spiked stool samples. A modified method of Chiu & Ou (1996) was used for spiking stool samples with different concentrations of *Salmonella* Typhimurium. A faecal specimen from a healthy individual was diluted 10-fold with BHI broth to minimize inhibition of PCR. This mixture was spiked with serial 10-fold dilutions of *Salmonella* Typhimurium culture by adding 250 μl of each diluted sample into 250 μl broth/faeces mixture and the spiked mixture was incubated at 37 °C for up to 6 h. The inoculated samples were sampled after 0, 4 and 6 h. PCR was then performed using boiled suspensions from the different time-points. The sensitivity of the PCR was defined as the lowest concentration of *Salmonella* Typhimurium (in c.f.u. ml^{-1} or per PCR) that yielded positive results. To rule out false positivity, a negative control containing unspiked faecal suspension was included in every experiment.

RESULTS AND DISCUSSION

The PCR produced an intense band of the expected 784 bp with all the *Salmonella* strains; none of the non-*Salmonella* strains gave any amplification, indicating 100 % specificity. Repeat PCR amplifications gave similar reproducible results. The limit of detection of the *hilA* gene was 100 pg in PCR using genomic DNA extracted from *Salmonella* Typhimurium ATCC 13311^T. A sensitivity of 3×10^4 c.f.u. ml^{-1} was observed when serial dilutions of bacterial cell culture were used as PCR template. This amount was equivalent to 120 c.f.u. per PCR (3×10^4 c.f.u. ml^{-1} in 4 μl).

As expected, the sensitivity of the PCR decreased to 3×10^5 c.f.u. ml^{-1} (1200 c.f.u. per PCR) in the presence of normal flora and inhibitors in the stool sample when direct stool samples were used as template in PCR (Fig. 1). However, after 4 and 6 h enrichment periods, the sensitivity increased to 3×10^4 c.f.u. ml^{-1} (120 c.f.u. per PCR) and 3×10^2 c.f.u. ml^{-1} (1·2 c.f.u. per PCR). Repeated PCR amplifications to test the sensitivity of the primers gave similar reproducible results.

During the process of *Salmonella* infection, invasion genes are required for bacterial entry into host cells. Many of these genes are encoded on *Salmonella* pathogenicity island 1 (SPI 1) (Mills *et al.*, 1995), which is present in all invasive strains of *Salmonella* (Galan, 1996) and absent from closely related genera such as *Escherichia* (Bäumler *et al.*, 1998; Mills *et al.*, 1995). The expression of these invasion genes is activated by *hilA*, a gene also encoded in SPI 1 (Bajaj *et al.*, 1995). The *hilA* gene is an important feature of *Salmonella* pathogenesis, as it is required for bacterial colonization of the extracellular, luminal compartment of the host intestine (Murray & Lee, 2000). Hence, in the present study, the specificity and sensitivity of a pair of primers targeting the *hilA* gene of *Salmonella* serovars (Cardona-Castro *et al.*, 2002) were assessed for the detection of *Salmonella* species in human faeces. The primers were originally designed with the intention of cloning an *hilA* gene fragment in order to disrupt this gene in the chromosome and elucidate its function in *Salmonella* species that are clinically important for humans (unpublished results). The forward primer contains a *Bam*HI site and has 17 nucleotides that match the *hilA* gene sequence perfectly. The reverse primer contains a *Hin*dIII site and 21

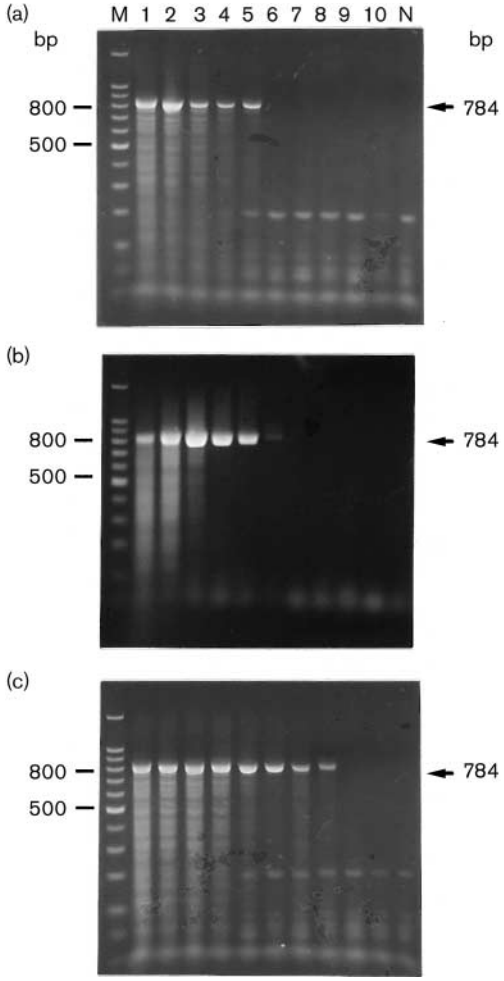

Fig. 1. Sensitivity of the PCR for detection of *Salmonella* Typhimurium ATCC 13311^T in faecal samples with pre-enrichment for 0 h (a), 4 h (b) or 6 h (c). Lanes: M, molecular size markers (100 bp DNA ladder); 1–10, faecal samples spiked with bacterial culture serially diluted 10-fold from 3×10^9 (lane 1) to 3×10^0 (lane 10) c.f.u. ml^{-1}; N, negative control (unspiked faecal sample as PCR template).

nucleotides match the *hilA* gene sequence perfectly. The primers did not need modification as they work perfectly for amplification of the *hilA* gene sequence in *Salmonella* species.

The most common problem in using a PCR assay for direct detection of an organism in faeces is that faecal samples contain substances such as bilirubin and bile salts that are inhibitory to PCR (Chiu & Ou, 1996; Stone *et al.*, 1994). This can be eliminated by DNA extraction from the faecal samples or by enrichment of the faecal samples in a suitable broth prior to PCR (Dutta *et al.*, 2001). However, several compounds used in DNA extraction procedures have been found to have some inhibitory effect on PCR (Rossen *et al.*, 1992). Some laboratories have described enrichment of faecal samples before performing PCR (Gentry-Weeks *et al.*, 2002; Stone *et al.*, 1994). However, in these protocols, DNA extraction from the enriched broth/faeces mixture is still required prior to PCR, using a commercially available kit, which is time-consuming and expensive. A magnetic immuno-PCR assay (MIPA) was devised for direct detection of salmonellae in human faeces (Widjojoatmodjo *et al.*, 1992). Although this procedure was rapid and sensitive, it was still expensive and was limited by the availability of antibodies (Chiu & Ou, 1996). Later, an enrichment broth culture-multiplex PCR combination assay was developed (Chiu & Ou, 1996). This method utilized the sequences of the *invA* and *spvC* genes of *Salmonella* bacteria in faecal samples. Faecal samples were diluted 10- to 20-fold prior to PCR in order to reduce the presence of PCR inhibitors.

A similar stool spiking and enrichment method (with some modifications) was used in the present study, with a single pair of primers targeting the *hilA* gene of *Salmonella* serovars. BHI broth, which is inexpensive and easy to prepare, was used for enrichment of *Salmonella*-spiked stool samples prior to PCR in order to eliminate inhibitors as well as to increase the sensitivity of the PCR assay. This method is simple and rapid, and results obtained in less than 18 h proved to be highly specific and sensitive. Although multiple bands of non-target size were occasionally observed in PCR products of *Salmonella* samples at lower dilutions of crude DNA template, this did not obscure the distinct and clear band of the expected size. The use of a hot staRT-PCR has been proposed to reduce non-specific priming (Roux, 1995).

In the present study, the faecal sample was diluted 10- to 20-fold prior to PCR in order to minimize the presence of PCR inhibitors. In the case of clinical specimens taken from patients, this will equally reduce the number of organisms present in the faecal samples. However, this will not affect the efficiency of the PCR assay, as it has proven to be highly sensitive.

Although we did not experience any problems with inhibitors in our spiked specimens, the method will have to be evaluated further on more faecal samples to ensure that the pre-enrichment step does eliminate inhibitory substances, since there may be specimen variation in levels and types of inhibitors. Our results confirmed that *hilA* gene-targeting primers are specific for *Salmonella* and that the PCR assay presented here is a promising technique for diagnosing infections with salmonellae using clinical specimens as well as for detecting carriers of *Salmonella* species.

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