Validation of a PCR for diagnosis of typhoid fever and salmonellosis by amplification of the hilA gene in clinical samples from Colombian patients

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Validation of a PCR test to detect hilA gene sequences of Salmonella spp. was performed in blood and faeces samples from typhoid fever and salmonellosis patients. Sensitivity (S), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) of the PCR in blood samples were performed by testing: 37 patients with clinical diagnosis of typhoid fever, 34 of them confirmed by isolation of S. Typhi from blood cultures; 35 patients infected with other pathogens corroborated by blood culture (Klebsiella pneumoniae, 9; Serratia marcescens, 5; Escherichia coli, 4; Pseudomonas aeruginosa, 9; Providencia alcalifaciens, 4 and Enterobacter cloacae, 4) and blood samples from 150 healthy volunteers. To evaluate S, SP, PPV and NPV of the PCR in faeces samples we studied: 34 patients with enteritis due Salmonella spp. (S. Typhimurium, 21; S. Enteritidis, 9; S. Choleraesuis, 3 and S. Agona, 1); faeces samples from 35 patients with enteric infection due to Shigella sonneratii (8), Shigella flexneri (10), enteropathogenic E. coli (12), Aeromonas hydrophila (5) and faeces samples from 150 healthy volunteers. The S, SP, PPV and NPV of the PCR in blood samples were all 100 %. PCR detected three patients with clinical diagnosis of typhoid fever and negative blood cultures. In faeces samples, S was 97 %, SP 100 %, PPV 100 % and NPV 99 %. The lowest number of c.f.u. ml⁻¹ detected by PCR in blood samples was 1 × 10³ and in faeces samples 4 × 10².

Introduction
Typhoid fever and salmonellosis are public health problems in developing countries, where the incidence of cases per year is 200–500/100 000. Transmission occurs by contamination of water or food with bacteria. Animals and humans are the principal reservoirs. Salmonella Typhi and Paratyphi A, B, C produce enteric fever only in humans, which is a severe infection that may progress to complications and death. Other Salmonella serovars produce enteritis in humans, and many animal reservoirs have been implicated in transmission: domestic and wild animals, reptiles, birds and insects. Salmonella cultures take 4–7 days for isolation and identification, a problem for diagnosis and treatment. In addition, sensitivity of cultures can be affected by antibiotic treatment, inadequate sampling, variations of bacteraemia and a small number of viable organisms in faeces (Miller & Pegues, 2000).

The development of molecular methods for diagnosis of infectious diseases has improved the sensitivity, specificity, quality and availability of diagnosis and treatment. We developed a PCR test to detect hilA, a regulatory gene found in pathogenicity island 1 of Salmonella spp. hilA is important for the regulation of the type III secretion apparatus, which is involved in the invasion of enterocytes (Lostroh et al., 2000; Mirol et al., 2001). hilA was detected by PCR and hybridization techniques in Salmonella enterica serovars Typhi, Pullorum, Choleraesuis, Enteritidis, Typhimurium (Cardona-Castro et al., 2002), Montevideo (Guo et al., 2000), and is not present in other bacteria (Pathmanathan et al., 2003).

Methods
Sample size and data analysis. The number of patients tested was calculated according to the prevalence of the disease in the region: 0·01 %, confidence 90 %, alpha error 0·05 and beta error 0·05 (Epi info 6 software program).

Sensitivity (S), specificity (SP), positive predictive value (PPV) and
negative predictive value (NPV) were calculated using Baye’s Theorem (Jekel et al., 1996). Briefly, three categories of patients were studied: (i) a true-positive patients group composed of 37 patients with clinical diagnosis of typhoid fever, 34 of them confirmed by isolation of S. Typhi from blood culture, and 34 patients with diarrhoea due to non-Typhi Salmonella isolated by faeces culture (S. Typhimurium, 21; S. Enteritidis, 9; S. Choleraesuis, 3; S. Agona, 1); (ii) a false-positive patients group with non-Salmonella-infected patients with similar symptoms, composed of 35 patients with other bacterial infection confirmed by isolation from blood culture (Klebsiella pneumoniae, 9; Serratia marcescens, 5; E. coli, 4; Pseudomonas aeruginosa, 9; Providencia alcalifaciens, 4 and Enterobacter cloacae, 4), 35 patients with diarrhoea due to other bacteria confirmed by faeces culture (Shigella sonnei, 8; Shigella flexneri, 10; entero pathogenic E. coli, 12 and Aeromonas hydrophila, 5) and (iii) a negative group with 150 blood and 150 faeces samples from healthy people. The gold standard test to compare the performance of the PCR method were blood and faeces cultures. The patients were admitted at several hospitals of the region and Instituto Colombiano de Medicina Tropical. Every patient, or tutor in the case of children, signed an informed consent. On the day of admittance, blood from typhoid-fever-suspected patients and faeces samples from patients with diarrhoea were taken to perform different procedures and PCR detection at the same time.

**Patients.** Volunteer patients of any age or sex, admitted with clinical suspicion of typhoid fever or diarrhoea, during the years 2001–2003, were included in the present study until the sample size was complete. The patients were admitted at several hospitals of the region and Instituto Colombiano de Medicina Tropical. Every patient, or tutor in the case of children, signed an informed consent. On the day of admittance, blood from typhoid-fever-suspected patients and faeces samples from patients with diarrhoea were taken to perform different procedures and PCR detection at the same time.

**Blood cultures.** Triplicate blood cultures were performed for each patient with 15 min or more between each testing. A proportion of 1:10 sample/broth was inoculated in trypticase soy (Becton Dickinson) and 0.05% SPS (sodium polyanethole sulfate) anticoagulant. Blood cultures were incubated at 37°C; inspection of bottles for turbidity, haemolysis, clotting or gas formation was made for at least 7 days. Gram’s staining reaction was done for blood cultures suspected of being positive. According to morphology and Gram reaction, subcultures were performed as recommended by Isenberg (1992).

**Faeces cultures.** Faeces samples were collected in a sterile screw-cap cup. Direct examination of fresh stool was done to observe parasites, PMN and erythrocytes. Faeces were inoculated in selenite F broth (Becton Dickinson) at 37°C for 12 h. Cultures in sorbitol MacConkey and Deoxycholate agar (Becton Dickinson) were performed (Isenberg, 1992).

**Bacterial identification.** Biochemical identification for Gram-negative bacilli was done using API 20E (bioMérieux). Serological identification of Salmonella spp. was performed at Instituto Nacional de Salud, Bogotá, Colombia.

**Inoculation of blood and faeces samples.** To determine the number of c.f.u. ml⁻¹ that the PCR method could detect, 10 blood and 10 faeces samples from healthy volunteers were inoculated with standardized inocula. S. Typhi S008 and S. Typhimurium ICMT02, were used to inoculate blood and faeces samples, respectively. One colony of an overnight culture of the bacteria on nutritive agar was inoculated in 1 ml of BHI (Becton Dickinson) and incubated at 37°C for 2 h, it was then diluted 1:10 in 9.85% saline solution. Absorbance of the dilution was measured at 640 nm, this reading became the basis for reproducing the inocula later. Serial dilutions of the 1:10 inocula were made up to 1:10⁻⁸, the number of c.f.u. ml⁻¹ per dilution was determined on nutrient agar (Becton Dickinson). This procedure was carried out each time the inocula were prepared. Five millilitres of fresh blood sample was inoculated with 1 ml of each dilution of S. Typhi inocula; 1 g of fresh faeces sample was inoculated with 1 ml of each dilution of S. Typhimurium inocula.

**DNA extraction.** Salmonella DNA from both clinical blood and artificially inoculated blood samples was obtained using the modified protocol of lysis buffer proposed by Haque et al. (1999). Briefly, 1 ml of blood sample was centrifuged at 10,000 r.p.m. for 5 min. The pellet was mixed with 1 ml 10 mM Tris/HCl, pH 8, 1 mM EDTA, 0.2% Triton X-100 and centrifuged at 12,000 r.p.m. for 6 min, this procedure was performed twice. After the second time, the pellet was mixed with 1 ml distilled water and centrifuged for 1 min at 12,000 r.p.m., 30 μl distilled water was added to the pellet followed by incubation at 100°C for 20 min. The sample was left at 4°C before PCR.

Extraction of the bacterial DNA from both patient and artificial faeces samples was performed according to the method described by Frankel et al. (1990). A swab of patient faeces samples and 0.5 ml of artificial inoculated faeces mixture was mixed with 4 ml PBS, pH 7.4, and then left for 15–30 min for the solid components to sediment; 1 ml of the supernatant was centrifuged at 16,000 g for 2 min. The pellet was mixed with 75 μl 50 mM Tris/HCl, pH 8.0, 20% (w/v) sucrose, 50 mM EDTA and 100 μl 100 μg lysosome ml⁻¹, then incubated at 37°C for 30 min. A 300 μl volume of 50 mM NaCl, 1% (w/v) SDS and 100 μl 800 μg proteinase K ml⁻¹ was added followed by incubation at 37°C for 50 min. The mixture was centrifuged at 14,000 g for 5 min. A 300 μl aliquot of the supernatant was taken. DNA was precipitated with absolute ethanol and centrifugation at 16,000 g for 1 min. The pellet was diluted in 500 μl Tris/EDTA (10 mM Tris/HCl, pH 8, 1 mM EDTA), before PCR.

**Primers.** The primers were designed according to the hilA gene sequence found at GenomeNet (www.genome.ad.jp), accession number U25352: US: 5’-GCATGGATCCTCGGGCGGAGATTGTTG-3’; DS: 5’-CGGAACGTATTTGCGCATGCTGAGGTAG-3’.

**PCR protocol.** The same protocol of amplification was used for PCR of blood and faeces samples. A 50 μl reaction volume was used, containing 1.5 μl each primer at 20 μM, 10 μl DNA, 3 μl 0.5 U Tag polymerase μl⁻¹ (Promega), 5 μl 10× buffer (Promega), 6 μl 20 mM MgCl₂, 0.1 μl each deoxynucleoside triphosphate at 20 μM (Promega) and 22.6 μl distilled water to complete the reaction volume.

PCR was performed in a programmable thermal controller (PTC-100; MJ Research). Parameters for amplification were as follows: initial denaturation at 94°C for 3 min, 30 cycles of 1 min each at 94°C, 65°C and 72°C and a final extension step at 72°C for 10 min. PCR amplions were electrophoresed in a 2% agarose gel. After staining with ethidium bromide, the amplified fragments in the gel were visualized. The molecular mass marker used was φX174 DNA/HaeIII markers (Promega). PCR was carried out in duplicate for each sample. One positive control (DNA from S. Typhimurium) and two negative controls (one without DNA and the other with DNA from a clinical isolate of E. coli) were included. A product of 854 bp was considered the hilA gene.

**Results and Discussion**

Table 1 shows the clinical characteristics, bacteriologic isolates and PCR results of the studied population. S, SP, PPV and NPV of the PCR test in blood samples were all 100%. In faeces samples S was slightly lower at 97%, because one of the 35 patients with diarrhoea due to Salmonella spp. was PCR-negative. SP and PPV were 100% and NPV 99%. Figs 1 and 2 show the 854 bp amplicon of hilA gene from blood and faeces samples positive for Salmonella spp. Detection of bacteria in the artificially inoculated blood and faeces samples showed that the PCR method could detect 1 × 10⁸ c.f.u. ml⁻¹ in blood samples and 4 × 10⁸ c.f.u. ml⁻¹ in faecal samples.
Several PCR methods to detect *Salmonella* spp. in food and human samples have been developed with the aim of improving diagnosis of the infection. Carli *et al.* (2001) used a combination of tetrathionate broth enrichment, capillary PCR and gel electrophoresis to detect *Salmonella* in chicken faeces. Chiu & Ou (1996) detected *Salmonella* in faeces using the virulence genes *invA* and *spvC* as targets for an enrichment broth culture-multiplex PCR. Most methods have been directed against all *Salmonella*, but Haque *et al.* (1999) developed their nested PCR method specifically to detect *S*. *Typhi*. Guo *et al.* (2000) developed a PCR to detect *S*. *Montevideo* in artificially infected tomatoes after enrichment, using two pairs of primers to amplify the *hilA* gene, as used in the current study. The HILA1 pair used by Guo *et al.* (2000) produced a PCR amplicon of 972 bp, but yielded non-specific bands with *Yersinia enterocolitica*; the HILA2 pair gave a PCR amplicon of 497 bp. Compared to the current study, the PCR method of Guo *et al.* (2000) thus uses combined techniques that increase both time and cost for the laboratory.

The PCR method in the current assay used a pair of primers that were originally designed with the intention of detecting the *hilA* gene in *Salmonella* species that are clinically important for humans (Cardona-Castro *et al.*, 2002). The forward primer contains a BamHI site and has 17 nucleotides that match the *hilA* gene sequence exactly. The reverse primer contains a HindIII site and 21 nucleotides match the *hilA* gene sequence exactly. The method is specific for *Salmonella*, simple and rapid; it does not use combined techniques, nor does it require enrichment culture before use and does not use a second amplification or nested PCR, taking less time and being cheaper.

According to the results, the current PCR method was more sensitive than blood culture, as it produced a positive test result from three patients with clinical diagnoses of typhoid fever. Table 1. Clinical characteristics, bacteriologic isolates and PCR results of the studied population

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. patients</th>
<th>Sample</th>
<th>Bacteriological isolation</th>
<th>No. PCR positive</th>
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</thead>
<tbody>
<tr>
<td>Typhoid fever</td>
<td>34</td>
<td>Blood</td>
<td><em>S</em>. <em>Typhi</em></td>
<td>34</td>
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<tr>
<td></td>
<td>3*</td>
<td>Blood</td>
<td>Negative blood cultures</td>
<td>3</td>
</tr>
<tr>
<td>Nosocomial pneumonia</td>
<td>9</td>
<td>Blood</td>
<td><em>K</em>. <em>pneumoniae</em></td>
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<td>Neonatal sepsis</td>
<td>5</td>
<td>Blood</td>
<td><em>Serratia marcescens</em></td>
<td>0</td>
</tr>
<tr>
<td>Sepsis due to infection of surgical wound</td>
<td>4</td>
<td>Blood</td>
<td><em>E</em>. <em>coli</em></td>
<td>0</td>
</tr>
<tr>
<td>Non-symptomatic healthy volunteers</td>
<td>150</td>
<td>Faeces</td>
<td><em>S</em>. <em>Typhimurium</em></td>
<td>21</td>
</tr>
<tr>
<td>Acute diarrhoea syndrome</td>
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<td>Faeces</td>
<td><em>S</em>. Enteritidis</td>
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</tr>
<tr>
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<tr>
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<td><em>Shigella flexneri</em></td>
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<td>Negative faeces cultures</td>
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<tr>
<td>Non-symptomatic healthy volunteers</td>
<td>150</td>
<td>Faeces</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Patient 1, female, 34 years old, received chloramphenicol 500 mg p.o. 2 days before hospital admittance. Patient 2, female, 18 years old, received TMS 80 mg/SMX 400 mg b.i.d. p.o. 3 days before hospital admittance. Patient 3, male, 60 years old, was admitted with intestinal perforation after 15 days with fever of unknown origin.

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**Fig. 1.** PCR of positive and negative faeces samples for *Salmonella* spp. M, Molecular mass marker (φX174 DNA/HaeIII) fragments; -, negative control; M1–M3, negative samples; +, positive control *hilA* gene; M4–M7, positive samples for *S*. *Typhimurium.*

**Fig. 2.** PCR of positive blood samples for *Salmonella* spp. –, Negative controls; M1–M8, positive blood samples for *S*. *Typhi*; +, positive control; M, molecular mass marker (φX174 DNA/HaeIII) fragments.

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fever, but with negative culture results; two of the patients had received antibiotic therapy before hospital admittance (Table 1). The results show that PCR methods can be an alternative tool to confirm clinical diagnosis in patients.

Inter-laboratory testing of methods to study the accuracy and robustness of PCR-based methods is an important step towards standardization and approval of the methods (Malorny et al., 2003). The current PCR method was tested in Malaysia, and showed good performance (Pathmanathan et al., 2003). However, the involvement of other endemic regions in testing the method is necessary to evaluate its use in clinical laboratories, and should be a future priority. Hoorfar et al. (2003) recommend the use of an internal amplification control (IAC) in order to improve the standardization of non-commercial PCRs and be confident that negative results are not false-negatives. The current study lacked an IAC, and theoretically, negative test results, e.g. from the negative patient group, could be false-negative. The method showed only one negative test among the positive samples, a faeces sample from a Salmonella-positive patient, possibly because of inhibitory substances in the sample. However, the good performance on positive blood and faeces samples, and the specificity of the PCR primers reported based on testing of other Enterobacteriaceae (Pathmanathan et al., 2003), indicate that the method has the required characteristics with regard to specificity and sensitivity. Future studies of application using this PCR method must include an IAC in order to improve standardization.

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References


