Detection of Rfbj (B) Gene of Salmonella Serogroup-B Isolated from Patients with Salmonellosis

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Summary

A total of 480 fecal samples were collected from patients (less than 18 years old), of both sex suffering from diarrhea who admitted to Al-Diwaniya Teaching Hospital and the Teaching Hospital of Maternity and Pediatrics in Al-Qadisiya governorate. Salmonella spp. was isolated and identified using bacterial culturing on selective media, in addition to, biochemical and serotyping by monovalent antisera. Polymerase Chain Reaction (PCR) was used to detect rfbj (B) gene encoding for biosynthesis of LPS of group B Salmonella. The results revealed that the rate of Salmonella isolates in fecal samples of patients were 7.9% using cultural and biochemical methods. The result of Salmonella isolates by serotyping using monovalent antisera revealed that 30 out of 34 isolates (88%) belong to S. Typhimurium serotype, while the remain belong to S. Enteritidis (2 isolates) and S. Muenchen (2 isolates). when the PCR technique was used to detect the presence of rfbj(B) gene, 34 Salmonella isolates belong to Salmonella serogroup (B) appeared to contain this gene since DNA amplification showed one distinct band (882 bp) when electrophorised on agarose gel. The results of this study revealed that the PCR technique had a high specificity (100%) in detection of Salmonella serogroup B in comparison to cultural, biochemical and serological tests.

Introduction

Salmonellosis is one of the most common infectious diseases in the world in both humans and animals (1). Salmonella are among the leading causes of community acquired food borne bacterial gastroenteritis worldwide (2). More than one-third of salmonellosis cases occur in children younger than 10 years old, and the incidence in children younger than 1 year old is 10 times higher than in the general population (3). Traditional Salmonella detection methods are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests. These methods are generally time-consuming, therefore, a rapid method is necessary for the identification of Salmonella serotypes from clinical specimens (4). There has been a general move toward molecular methods of Salmonella detection and typing, which are based less on phenotypic features and more on stable genotypic characteristics (5). PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy (6). Serotyping of Salmonellae is a valuable phenotypic subtyping tool for understanding the epidemiology of this important foodborne pathogen (7). Serotyping have limitations; production and quality control of the hundreds of antisera required for serotyping is difficult and time consuming, and not all Salmonella strains are typeable (8). Salmonella isolates are serotyped using the Kauffmann-White scheme according to their O, H, and Vi antigens (9,10). Many of the genes required for O-antigen biosynthesis are organized in a large regulon termed the rfb gene cluster (11). rfb gene clusters have been
characterized from a growing number of gram-negative bacteria; this operon is located between galF and gnd in *Salmonella enterica* and *Escherichia coli*\(^{(12)}\). Typically, three classes of genes are found in *rfb* clusters: (i) genes for synthesis of nucleotide sugars specific to the respective O antigen, (ii) sugar transferase genes to build the O subunit, and (iii) the O-antigen polymerase (*wzy*) and transport protein (*wzx*) genes for assembly of the O subunit into the O antigen\(^{(12)}\). The aim of study was to evaluate the rapid diagnostic test to identify salmonellosis using PCR technique for the detection of *rfbj(B)* gene to differentiate among *Salmonella* serogroups.

**Materials and Methods**

-Samples Collection: A total of 480 stool samples were collected from inpatients and outpatients (both sex) with diarrhea who were admitted to Al-Diwaniya Teaching Hospital and Al-Diwaniya Teaching Hospital for Maternity and Children. One gram of stool sample was placed in 5 ml of Selenite broth, labeled and transported to the laboratory in portable container, then incubated for 18-24 hrs at 37°C\(^{\circ}\)\(^{(13)}\). This study was conducted during the period that extended from November 2008 to October 2009.  

**Identification of *Salmonella* spp.:** After culturing on Selenite broth, a loopful of broth was streaked on surface of *Salmonella*-Shigella agar (S.S), Xylose-lysine deoxycholate (XLD) and Brilliant green agar (BGA) plates and then incubated at 37°C for 24 hrs. The biochemical characters of non-lactose fermenting bacteria was determined by using Triple Sugar Iron agar (TSI) and Urease test \(^{(13)}\). Colonies that showed biochemical characteristics similar to that of *Salmonella* spp. were tested by serogrouped using monovalent antisera in central health laboratory/ Baghdad and the confirmation was identified by PCR with *rfbj(B)* genes primers for detection of *Salmonella* serogroup B\(^{(14)}\).

**PCR method: DNA Extraction and Purification:** The DNA of all isolates were extracted using genome DNA purification kit.(DNA-sorbB) provided by Sacacebiotechnologies, Italy. **Primers:** The primers were used for the detection of *rfbj (B)* gene coding for biosynthesis of lipopolysaccharide (LPS) of group B *Salmonella* according to\(^{(14)}\), which is provided by Alpha DNA company (Canada)(Table 1).

**The *rfbj (B)* Gene Detection:**

For the detection of *Salmonella* serogroup B by PCR the specific primers of *rfbj (B)* gene which is responsible for biosynthesis of lipopolysaccharide (LPS) of *Salmonella* serogroup B were used. The PCR amplification mixture (25µl) which was used for the detection *rfbj(B)* gene includes 12.5 µl of (Green master mix, 2x,which provided by promega ,U.S.A )include: Taq DNA polymerase; dNTPs which include: 400 µM of each dATP, dGTP, dCTP, dTTP; 3mM of MgCl\(_2\);Yellow and blue dyes as loading dye), 2.5 µl of template DNA , 1.25 µl of each forwarded and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to 25 µl. The PCR tubes containing amplification mixture were transferred to preheated thermocycler and started the program as in the following reaction was taken through 25 cycles in a DNA thermal cycler. Each cycle consisted of the

**Table 1. Specific primers used for the detection specific sequence of *rfbj (B)* gene.**

<table>
<thead>
<tr>
<th>Nucleotide sequence</th>
<th>position</th>
<th>Tm(°C)</th>
<th>Size of PCR products(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 5’-AGAATATGTAATTGTCAG-3’</td>
<td>15-32</td>
<td>48</td>
<td>~882</td>
</tr>
<tr>
<td>Reverse 5’-TAACCGTTTCAGTAGTTTC-3’</td>
<td>880-897</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>
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following: 94°C for 1 min (denaturation), 45°C for 1 min (annealing), and 72°C for 2 min (extension). After PCR, the profiles of amplification products were detected by gel electrophoresis. Ten microliters of total reaction mixture was loaded on a 2% agarose gel and electrophoresed at 110V at 70 mA for 45 to 60 min. The amplified DNA fragments were visualized by UV illumination after agarose gel electrophoresis and ethidium bromide staining (14).

Amplification of target DNA (rfbj(B) gene): The PCR amplification which was performed on the DNA extracted from all the studied isolates were confirmed by electrophoresis analysis. The electrophoresis was also used to estimate DNA weight depending on DNA marker (100 bp DNA ladder).

Statistical Analysis

All results were performed by Chi square test at the level of significant when P-value < 0.01. The specificity, sensitivity and diagnostic accuracy of the results were also calculated by applying the following equations: (a / a + b) x 100 = sensitivity, (d / d + c) x 100 = specificity. diagnostic accuracy = (a+d / a+b+c+d)x100. also calculated positive predictive value (PPV) = (a / a + c)100 and negative predictive value (NPV) = (d / b + d)100. (a = the total number of positive cases, b = false positive those bearing positive reading from negative samples, d = total number of true negatives, c = those with negative reading from positive cases (15).

Results

Laboratory investigations: Culture, serotyping and PCR: The percentage of Salmonella spp. isolation was 7.9% (38/480) by using the conventional culture methods of stool samples on enrichment and selective media (Table 2). There was a significant differences (P<0.01) between the positive and negative results.

Table 2. Percentage of Salmonella spp. isolation by using culture methods and biochemical tests.

<table>
<thead>
<tr>
<th>Isolation results</th>
<th>No.</th>
<th>%</th>
<th>X^2 value (p&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive results</td>
<td>38</td>
<td>7.9</td>
<td>Calculated X^2=340.033</td>
</tr>
<tr>
<td>Negative results</td>
<td>442</td>
<td>92.1</td>
<td>Tabulated X^2=6.6349</td>
</tr>
<tr>
<td>Total</td>
<td>480</td>
<td>100</td>
<td>df= 1 (significant)</td>
</tr>
</tbody>
</table>

Out of the 38 cases of the study group, 38 cases (100%) gave positive results for culturing and biochemical tests, 34 cases (89.5%) gave positive results for serotyping and PCR tests and 4 cases (10.5%) gave negative results for serotyping and PCR. In this study, 12 cases of non-Salmonella spp. (control group) gave negative results for culture, serotyping and PCR (Table 3).

Table 3. Different laboratory techniques used for Salmonella spp. and non-Salmonella isolates.

<table>
<thead>
<tr>
<th>Test</th>
<th>Culture</th>
<th>serotyping</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Study group</td>
<td>38</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Non Salmonella isolates</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

PCR Results: The result of the amplified DNA was 882 bp when compared with 100bp marker.
Comparison of PCR technique and Other Used Tests for Detection of *Salmonella* spp.; Out of 38 cases that gave positive results as *Salmonella* spp on culture media and biochemical tests, 34 cases (89.5 %) were positive for serotyping and PCR techniques and 4 cases (10.5 %) were negative for PCR and serotyping tests (Table 4). Out of 12 cases that gave negative results in culture and biochemical tests, these 12 cases (100%) gave negative for PCR, serotyping tests. There were significant differences (P<0.01) between the PCR and culture media methods and serotyping in diagnosis *Salmonella* spp. (table 4).

Table 4. Comparison of PCR technique and other used tests for detection *Salmonella* spp. and *Salmonella* serogroup -B

<table>
<thead>
<tr>
<th>Tests</th>
<th>PCR method</th>
<th>Total</th>
<th>X² value (p&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
<td>No.</td>
</tr>
<tr>
<td>Culture method</td>
<td>34</td>
<td>89.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>serotyping</td>
<td>34</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

**PCR results Versus Selective Media and Biochemical Tests:** The sensitivity, specificity, accuracy PPV and NPV of PCR test were (89.5%, 100%, 92%, 100%, 75%) respectively while for culture and biochemical tests, there were (100%, 75%, 92%, 89.5%, 100) respectively.

**PCR results Versus Serotyping Test** The sensitivity, specificity, accuracy, PPV and NPV of the PCR and serotyping tests were 100% for all the above statistic parameter.

**Comparison between PCR and Serotyping tests for Detecting *Salmonella serogroup B***:
Out of 34 cases from *Salmonella* spp. that gave positive on serotyping, there were 30 cases positive for *Salmonella* serogroup B and 4 cases (2 cases S. Enteritidis and 2 cases S. Meunchen). In PCR, there were 34 cases positive to group B *Salmonella* which gave positive results to *rflj*(B). Out of 16 cases that gave negative results in serotyping also gave
negative results for PCR test. There were significant differences (P<0.01) between the PCR and serotyping methods in diagnosis *Salmonella* serogroup B (Table 5).

Table 5. Relationship between PCR and serotyping for detection *Salmonella* serogroup B

<table>
<thead>
<tr>
<th>Tests</th>
<th>PCR method</th>
<th>Total</th>
<th>$X^2$ value (p&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No. %</td>
<td>No. %</td>
<td></td>
</tr>
<tr>
<td>PCR method</td>
<td>30 100</td>
<td>0 0</td>
<td>Cal.$X^2$=35.29</td>
</tr>
<tr>
<td>Serotyping method</td>
<td>4 0</td>
<td>16 100</td>
<td>Tab.$X^2$=6.634 (Significant)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>df=1</td>
</tr>
</tbody>
</table>

**PCR Results Versus Serotyping for Detecting *Salmonella* serogroup B:** The sensitivity, specificity, accuracy, PPV and NPV of PCR test were (100%, 80%, 92%, 88.2%, 100%) respectively while for serotyping, there were (88.2%, 100%, 92%, 100%, 80%) respectively.

**Discussions**

In this study, we found that *Salmonella* spp. infection in Al-Diwaniya Governorate is considered one of the causes of diarrhea. This may reflect the fact that *Salmonella* spp. is one of etiologic agents of diarrhea that infect infants and young adults especially during the summer, and that *Salmonella* is a zoonotic bacterial agent, and *S. Typhimurium* is the most common serotype found in animals and in humans\(^{(16)}\). In this study, we found 38 (7.9%) suspected isolates of *Salmonella* spp. on culture and biochemical tests. Other studies also conducted in Al-Diwaniya province revealed the prevalence of *Salmonella* spp.: 10%, 14.47%, 8.47% which were reported by, \(^{(17,18,19)}\) respectively. *Salmonella* detection in stool using conventional media, such as *Salmonella-Shigella* agar (SS), is based on lactose fermentation and H2S production. The number of false-positive results with these media necessitates time-consuming and expensive additional testing\(^{(20)}\). The study revealed that significant differences (P < 0.01) between conventional methods and PCR for diagnosis of *Salmonella* spp. and the PCR is more specific than conventional method, because the conventional methods for *Salmonella* spp., have very poor specificity, and there were numerous false- positive results\(^{(21,22)}\). Furthermore, serotyping of isolates by monospecific antisera, we found 30 (88 %) *S. Typhimurium* isolates, 2 (6 %) *S. Enteritidis* and 2 (6 %) *S. Meunchen*; while according to the results of PCR, 32 isolates (94 %) were *S. Typhimurium* and 2 isolates (6 %) belong to *Salmonella* group B but no *S. Typhimurium*. The results revealed that there were significant differences between PCR and serotyping for the diagnosis of *Salmonella* serogroup B and we found PCR is more specific than serotyping in the diagnosis of *Salmonella* serogroup B, while serotyping is the method of choice to identify and discriminate isolates of *S. enterica*. But, the serotyping has a number of deficiencies, including the inability to serotype 5 and 8% of isolates and the incorrect typing due to the loss of surface antigens\(^{(23,24)}\). The results showed that all *Salmonella* spp. isolates 34(100%) belong to serogroup B by using specific primer set to confirm the isolation as positive *Salmonella* by detecting *rfbj*(B) gene. The selected *rfbj* gene sequences were proved to be useful DNA-based markers for identification and differentiation among *Salmonella* serogroups A, B, C2, and D\(^{(14)}\), all PCR products of isolates screened by PCR results in 882 bp amplified fragment.
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Non amplified DNA fragment were obtained from non Salmonella serogroup B.

Conclusions

1. The polymerase chain reaction (PCR) technique gave a high specificity in comparison with other done test, with its advantages of greater speed and effectiveness than conventional detection method; it was successfully used to identify the Salmonella spp
2. Using PCR technique for direct stool samples without Lab. culture procedure gave a good result in direct diagnosis of Salmonella spp.
3. We can classify Salmonella isolates based on, rfbj (B) gene as Salmonella serogroup B.

Recommendations

1. PCR is very suitable, highly specific, test and can be used as a basis for future application.
2. Further study is suggested to test different version of the PCR methods using different samples, to select the most sensitive and specific method.

References

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