DETECTION OF CLOSTRIDIUM BOLTEAE IN SAMPLES OF IRAQI AUTISTIC CHILDREN USING TRADITIONAL AND MOLECULAR METHODS

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Abstract

Clostridia are widely distributed in natural environments and are inhabitants of both human and animal gastrointestinal tract. These organisms are important pathogens that may cause pseudomembranous colitis, necrotizing enteritis, food poisoning and the other intestinal disorders, such as diarrhea. The objective of this study was to detect C. bolteae from samples of Iraqi autistic children. Stool samples were collected from children under ten years. The samples were subjected to culturing and DNA extraction. Three isolates were detected by traditional method and showed positive results, while in conventional PCR furthermore detection of target bacteria using RT-PCR that is more sensitive, more reliable and fast.

Key words : Clostridium bolteae, PCR, Autistic children.

Introduction

Up to our knowledge this is the first study in Iraq for detection of C. bolteae. Clostridium spp. are widely distributed in the environment, some of these Clostridia are inhabitants of human gastrointestinal tract and may cause intestinal disorders such as diarrhea, pseudomembranous colitis, necrotizing enteritis and food poisoning. (Ferreira et al., 2003). However, Lorber (2005) reported that most cases of clostridial infection are seen in patients with a history of trauma, recent surgery, diabetes, colon cancer, skin infections/burns and septic abortions. Delost (1997), Brazier et al. (2002) and Winikoff (2006) demonstrated that based on role of pathogenesis, Clostridia are classified in to (5) groups: Group I, related to myonecrosis or gas gangrene including: C. perfringens, C. septicum, C. novyi, C. bifermentans, C. histolyticum and C. sordellii. Group II which causes tetanus, including C. tetani, Group III, cause botulism, including C. botulinum, Group IV which causes acute diarrhea associated with antibiotic-associated and pseudomembranous colitis, including C. difficile, and Group V related to cerebral abscesses, abdominal and gynecologic infections, pneumonia and bacteremia, including: C. perfringens, C. ramosum, C. bifermentans.

Finegold et al. (2002) described C. bolteae as a member of the normal intestinal microbiota of humans, which can cause intra-abdominal infections when the natural intestinal barrier is altered, C. bolteae is present in stools of most children, but counts are significantly higher in autistic children than in controls. Furthermore, Pequegnata et al. (2013) confirmed that chronic diarrheal episodes associated with some forms of autism could be attributed to an overabundance of C. bolteae and release of end products of metabolism, such as butyrate, propionate and acetate, that alter the motility and contraction rate of the gastrointestinal tract. To test this hypothesis this study was designed for traditional isolation and molecular identification of Cl. bolteae from stool specimens of Iraqi autistic children.

Materials and Methods

Sample collection

A total of 80 stool samples were collected from autistic and healthy children (under ten years) in Baghdad city. A fresh stool sample was collected in a sterile container, once it has been collected, they were transferred to the laboratory immediately or refrigerated and taken to the lab as soon as possible.
Culturing procedure

Stool specimens were treated with 95% ethanol, (Moosavian and Hayati, 2008). Inoculate samples in thioglycolate broth overnight then 50μl from the bottom of each tube stretching on Brucell Blood Agar media and incubated at 37°C in anaerobic jar with Anaerogen gas back Kit and observed after (48-72) hrs. Gram stain and biochemical tests were conducted, then, they were identified by molecular methods (Shin et al., 2014).

Molecular analysis

DNA extraction

Genomic DNA was extracted from stool samples directly by employment of Genomic DNA Purification kit (Bioneer Korea). Bacterial genomic DNA was extracted from culture by employment of Genomic DNA Purification kit (Genaid Taiwan).

PCR detection

Amplification with conventional PCR was carried out in a total volume of 25μl containing PCR pre Mix (promega) Ready-to use (12.5μl): Forward primer (1μl) Reverse primer(1μl), (5μl) of DNA template and distal water (5.5μl). Optimal PCR program was carried out for 35 cycles, each cycle consisted of 95°C for 20s for denaturation, 58 °C for 1 min for annealing, 72°C for 30 s for extension and cycle of 72°C for 5 min was added to the final extension. PCR products were verified by bands seen through the ultraviolet (UV) transilluminator via ethidium bromide staining after carrying out electrophoresis using a 2% agarose gel and photographed.

Real time PCR

The assay was performed with the Rotor gene instrument in a protocol comprising 1 cycle of 2 min at 95°C (hot start) followed by 40 cycles of 95°C for 20 s for denaturation. Annealing was performed for 30s at 58°C. Extension was performed at 72°C for 45 s. And total reaction volume was 20μl composed of 1μl of each primer (forward and revers), 1μl of prob, 2μl of RNase–free water, 10μl RT-PCR GoTaq Master Mix, dTTP and 5μl template DNA. Data analysis was performed using Rotor-Gene software.

Results and Discussion

Isolation of C. bolteae

Phenotypic characterization

In this study, three isolates revealed colonies with 2–3mm in diameter, slightly scalloped edges, grey, circular, waxy, as shown in fig. (1).

Microscopic finding

Organisms appeared to be gram-positive, rod-shaped, typical rod were 1.0–1.2μm, 2.0–5.0 increasing in width and short when start to forming oval sub terminal spore. results as shown in fig. (2). The results of phenotype and Microscopic finding is coincides with Song et al. (2003).

Biochemical test

The presumptive C. bolteae isolates shown results coincides with Song et al. (2003) as in table 2.

Conventional PCR assay was applied to amplify 16S rRNA gene, which result in producing the predicted band with size of 150bp as shown in fig. (3).

The results suggested that the combination of sample processing with the high-performance identification method could be applicable for routine work used in clinical checking (Surang et al., 2013). The molecular approach has been largely performed in several laboratories, so that it is high sensitivity and specificity as well as rapid (Luo and Banaei, 2010; Luna et al., 2011).
Detection of *C. bolteae* in Samples of Iraqi Autistic Children

In this study, PCR findings was similar to that of Song *et al.* (2004), who firstly described the species and Song *et al.* (2005) who differentiate *C. bolteae* from other closely related species.

**Real time PCR**

The suitability of 16s rRNA gene was used for diagnosis of *C. bolteae* in stool specimens by Real-time PCR. Among 40 stool specimens collected from autistic children, 31 (77.50%) were positive for *C. bolteae* (fig. 4).

On the other hand, no amplification was observed in other (9) 22.50% while control group was 19 (47.50%) positive result and no amplification was seen in other 21 (52.50%). There was statistically significant differences (P<0.01) between the two groups (table 3).

The CT value is proportional to the amount of target DNA and hence the number of bacteria in the samples (Song *et al.*, 2004). The presence of fastidious bacteria including anaerobes make culture-based diagnosis challenging, real-time PCR offers a culture-independent method for bacterial quantification (Edith *et al.*, 2010).

As well as detection of products by real-time PCR occurs during the log phase instead of the plateau phase, which gives more accurate quantification of target concentration (Pau Ni *et al.*, 2006). As known, the efficacy of real-time PCR may return to the amplification of target DNA under short time condition (less than 2 hours) and to the quality of TaqMan PCR master mixes (Dagher *et al.*, 2004).

**Conclusion**

Molecular technique for quantifying anaerobic
Table 2: Biochemical results of presumptive *C. bolteae* isolates.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>–</td>
</tr>
<tr>
<td>Lechithinase production</td>
<td>–</td>
</tr>
<tr>
<td>Lipase production</td>
<td>–</td>
</tr>
<tr>
<td>Indole production</td>
<td>–</td>
</tr>
<tr>
<td>Esculin hydrolysis</td>
<td>–</td>
</tr>
</tbody>
</table>

Fermentation

| Glucose       | + |
| Fructose      | + |
| Glycerol      | + |
| Maltose       | + |
| Mannose       | + |
| Melezitose    | + |
| Sorbitol      | + |
| Sucrose       | + |
| Trehalose     | + |
| Arabinose     | + |

Table 3: Comparison between patients and control in CT.

<table>
<thead>
<tr>
<th>The group</th>
<th>No.</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>31</td>
<td>28.01 ± 0.47</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>29.91 ± 0.54</td>
</tr>
<tr>
<td>T-Test</td>
<td></td>
<td>1.492 **</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.0103</td>
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** (P<0.01).

bacteria and other microbiota is the best. The assay is rapid, reliable and time saving. A major advantage of this method is that it lends itself to high throughput.

References


