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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 transfered to the laboratory immediately or refrigerated and taken to the lab as soon as possible.

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Fig. 1 : Colonies of *C. bolteae* on brucella blood agar under anaerobic condition at 37°C for 48 hours incubation.

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 37ºC 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).

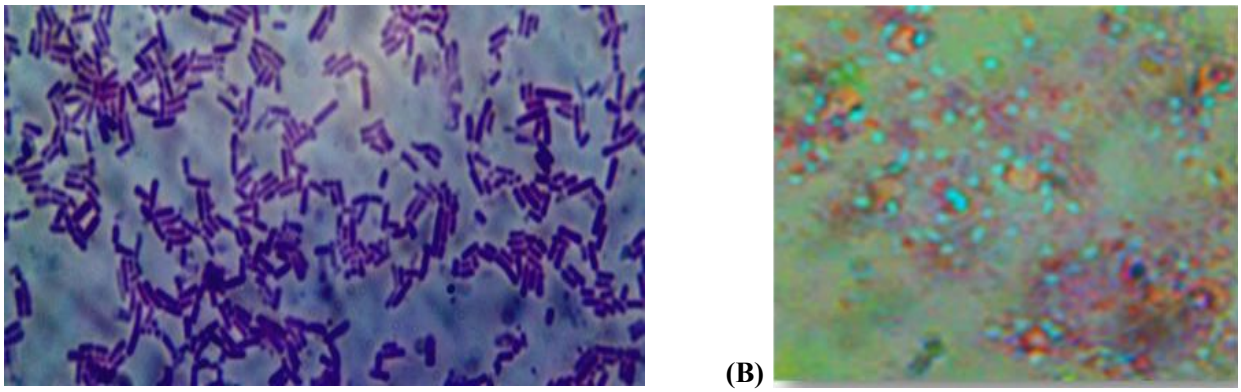

Fig. 2 : Rods of *C.bolteae* : A stained with Gram stain, B stained with Malachite green stain.

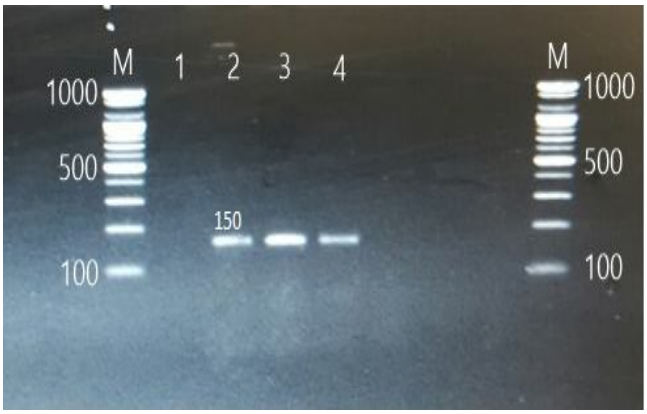

Fig. 3 : PCR products of *16S rRNA* genes, Lane 1 negative, control, Lanes 2-4: samples, Lane M: marker 2% agarose gel at voltage 100 for one hour.

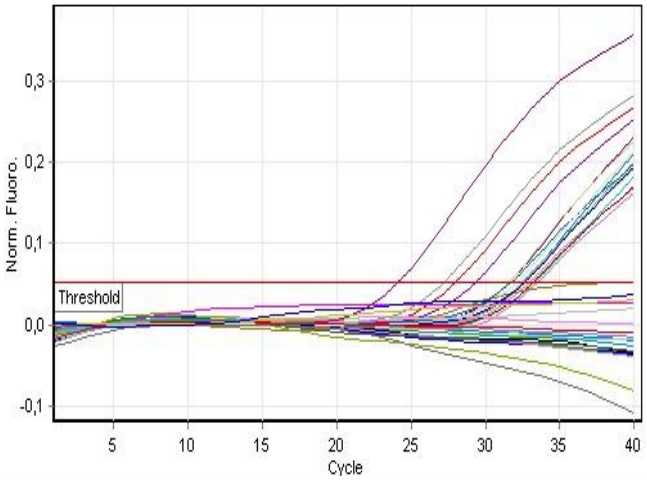

Fig. 4 : The Result of end point RT-PCR Run for *16SRNA* Gene.

Table 1 : Primers and prob used in this study.

Primer sequences	Amplicon size (bp)	Refrence
F: CCTCTTGAC CGGCGTGTR: CAGGTAAAGCTGGGCACTCTAGG	150	Yuli *et al.* (2002)
F: AGAGTTTGATCCTGGCTCAGR: GGTTACCTTGTTACGACTT	1,350	Ramya *et al.* (2014)
Prob: CAGGTGGTGCATGGTTGTCGTCAG		Ramya *et al.* (2014)

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 *16srRNA* 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.

Test	Result
Catalase	–
Lecithinase production	–
Lipase production	–
Indole production	–
Esculin hydrolysis	–
Fermentation	
Glucose	+
Fructose	+
Glycerol	+
Maltose	+
Mannose	+
Melezitose	+
Sorbitol	+
Sucrose	+
Trehalose	+
Arabinose	+

Table 3 : Comparison between patients and control in CT.

The group	No.	Mean ± SE
Patients	31	28.01 ± 0.47
Control	19	29.91 ± 0.54
T-Test	—	1.492 **
P-value	—	0.0103

** (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.

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