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PCR for Detection of *Clostridium difficile* toxin A (tcdA)and toxin B (tcdB) genes in Iraq

Luma Yousif Mehdi[1*] Mona Turkey AL-Mossawei[2]
1, College of Health and Medical Technology , Baghdad ,Iraq.
2, Biology Department , College of Science for Women ,Baghdad University , Iraq.

Abstract
Clostridium difficile is the most common cause of Antibiotic-Associated Diarrhea and Colitis, occurring after exposure to antibiotics . Although there are many diagnostic methods ,the nucleic acid-based approach has been largely performed in several laboratories, due to its high sensitivity and specificity as well as rapid . This is the first study in Iraq for detection of toxins A&B genes, and to focuses on diagnosis of *C. difficile* by PCR method to confirmed isolation were tested previously by culturing , Api20A and ELISA methods .Results show that all 75 positive isolation previously tested ,were having toxin A(tcdA) and toxinB(tcdB) genes . The results suggested that the combination of sample processing with the high-performance detection method would be applicable for routine diagnostic use in clinical setting.
Keywords: Clostridium difficile , PCR, Api20A , ELISA .

INTRODUCTION

Clostridium difficile is a motile, rod-shaped, Gram-positive bacterium, which is known to be a leading cause of antibiotic associated diarrhea, especially nosocomial infections [1]. Two large toxins, TcdA enterotoxin and TcdB cytotoxine(308 kDa and 270 kDa, respectively), are recognized as the main virulence factors of *C. difficile* disrupting tight junctions of the intestinal epithelial cells resulting in inflammation and increased permeability of the intestine [2]. The two genes are part of the PaLoc operon, which also contains tcdR, tcdE and tcdC, of which tcdC is a putative negative regulator of tcdA and tcdB [3]. *C. difficile* infection results in a wide range of symptoms including fever, abdominal pains, mild diarrhea, and pseudomembranous colitis ,the hypervirulent strains that are resistant to current therapy , are able to produce high titers of toxins poses a challenge to the treatment of infection worldwide [4]. *C. difficile* can be diagnosed by culturing stool samples on selective media, and toxigenic strains producing TcdA and ⁄ or TcdB may subsequently be identified by ELISA. Diagnostic strategies targeting nucleic acids, including PCR methods [5,6,7] and real-time PCR methods [8,9], have been developed for the detection of the genes encoding TcdA and ⁄ or TcdB. This is the first study in Iraq, that detection of toxin A and B by ELISA and PCR method.

AIM OF STUDY

The study was designed to evaluate PCR method to confirmation diagnosis of *C.difficile* by previous methods .

MATERIALS AND METHODS

Samples :
Four hundred thirty ,the total stool samples were collected during the period of first of June 2013 , till the end of April 2014, from Iraqi patient ,children and adults suffering from antibiotic associated diarrhea and colitis ,and apparently healthy children and adults as showing in table (1) .

Primary identification
Stool samples were streaked on selective media(CCFA) (Sigma Aldrich, UK)+7%horse blood as[10] ,incubation in anaerobic conditions at $37C^0$ for 48hrs ,and isolates were presumptively identified (Gram stain , and Malachite green for spore) ,definitive identification was performed by Api20A kit (BioMerieux ,USA), and detection of two toxins A& B in stool samples by ELISA Kit (Premier toxin A&B from Meridian Bioscience ,USA), according to the manufacturer's recommendations. Genomic DNA of all 75 positive isolation were tested by previously methods, were extracted and analyzed with PCR**.**
The aim of the present study was to use new molecular methods for conformation the detection of pathogenic *C. difficile* isolates, including PCR for detecting the genes encoding TcdA, TcdB.

Table (1): Distribution of study samples .

Age	Total No. of patient samples	Positive No. of patient isolates	Total No. of healthy samples	Positive No. of healthy isolates
Children (after birth -- 15 years)	**240**	**51**	**80**	**12**
Adults (16 to ≥ 61)	**70**	**11**	**40**	**1**
Total	**310**	**62**	**120**	**13**

DNA extraction:
Bacterial genomic DNA was extracted from BHI broth samples by employment of Genomic DNA Purification kit (Promega, USA).

PCR amplification: The primers used were tcdA (forward, , 5'-GCA TGA TAA GGC AAC TTC AGT GGT A--3' and (reverse, 5'- AGT TCC TCC TGC TCC ATC AAA TG-3') primer pairs (Alpha DNA, Canada) which were described previously [11,12].

PCR amplifications were carried out in total reaction volumes of 25µl containing 1 µl of each primer, 5µl of Master mix (Accu power® PCR PreMix ,BioNeer ,Korea), 6µl DNA template and 13 µl of ultra-pure distilled water.

The amplification thermo cycle parameters condition were 5min at $95C^0$,followed by 35 cycles of 30s at $95C^0$,1min at 53 C^0 and 1min at $72C^0$,and final extention of 5 min at $72C^o$. Expected products of amplification are 625bp[11].

The primers used were tcdB (forward, 5'-CCA AAG TGG AGT GTT ACA AAC AGG TG-3') and (reverse, 5'-GCA TTT CTC CGT TTT CAG CAA AGT A-3') primer pairs (Alpha, Canada) which were described previously [11,12].

PCR amplifications were carried out in total reaction volumes of 25µl containing 1 µl of each primer, 5µl of Master mix (Accu power® PCR PreMix, BioNeer ,Korea), 6µl DNA template and13 µl of ultra-pure distilled water.

The amplification thermo cycle parameters condition were 5min at $95C^0$,followed by 35 cycles of 30s at $95C^0$,1min at 56 C^0 and 1min at $72C^0$,and final extention of 5 min at $72C^o$. Expected products of amplification are 410bp[11].

Samples were loaded 7 µl carefully into the individual wells of gel(1% w\v agarose, Promega ,USA)stained with ethidium bromide (5 µl \100ml), and then electrical power was turned on at 70volt for 1 hrs .

Results and Discussion

Clostridium difficile is the most common causative agent of primary and recurrent antibiotic-associated diarrhea and colitis in hospitalized patients[13]. The disease is caused mainly by two exotoxins, TcdA and TcdB, produced by the bacteria. The symptoms of *C. difficile* infection range from asymptomatic colonization to mild diarrhea and severe life threatening pseudomembranous colitis, a cause of antibiotic-associated diarrhea (AAD), is an inflammation of the colon. Therefore, a rapid and cost effective method to detect *C. difficile* directly from stool samples facilitates patient management to control infection.[14,15].

Seventy-five strains contained both tcdA (figure 1) and tcdB(figure2) genes .Pathogenic strains of *C.difficile* produce tcdA and tcdB ,and several strains isolated from outbreaks and severe infection have been shown harbour the genes encoding toxins.

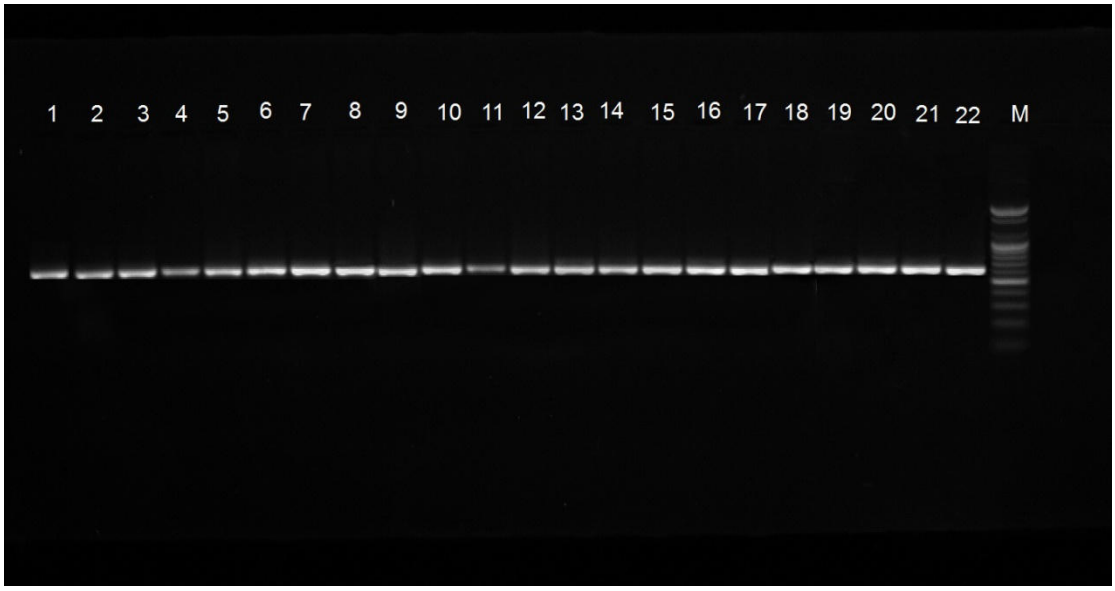

Figure (1): Agarose gel electrophoresis of PCR amplification products of *C.difficile* Toxin A (tcdA)gene .Expected products of amplification are 625bp.

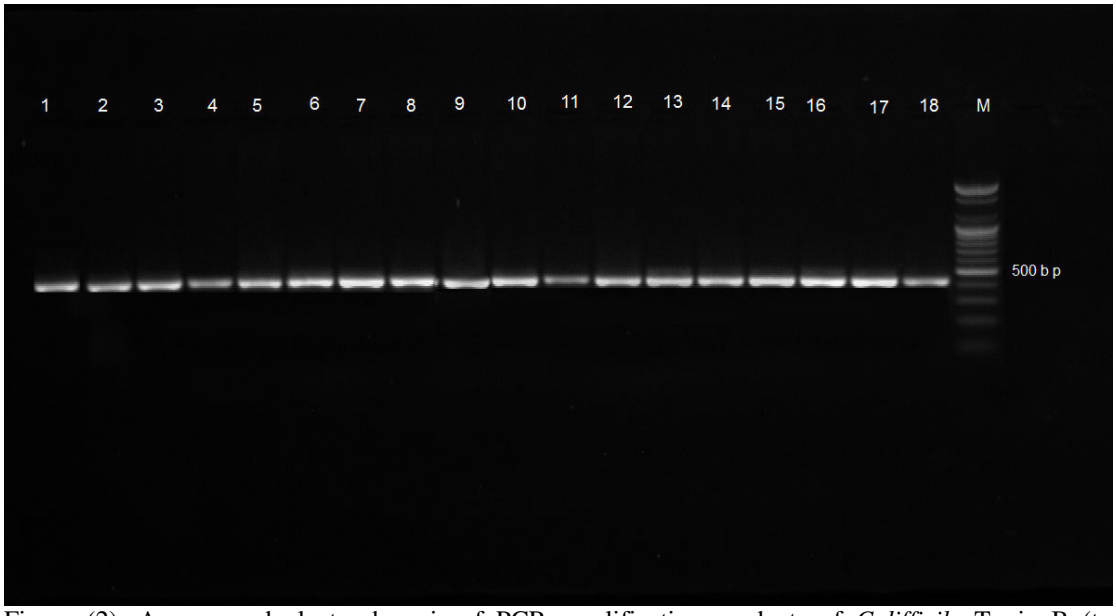

Figure (2): Agarose gel electrophoresis of PCR amplification products of *C.difficile* Toxin B (tcdB)gene .Expected products of amplification are 410bp.

This is the first study in Iraq, that detection of toxin A and B genes by PCR . This rapid and cost-effective diagnostic assay provides an alternative approach for the detection of *C. difficile*, thereby improving the CDI management.

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