

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

Luma Yousif Mehdi<sup>1\*</sup> Mona Turkey AL-Mossawei<sup>2</sup>

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 focus 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 toxin B(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 37°C for 48 hrs, 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 confirmation 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
<b>Total</b>	<b>310</b>	<b>62</b>	<b>120</b>	<b>13</b>

**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<sup>0</sup>,followed by 35 cycles of 30s at 95C<sup>0</sup> ,1min at 53 C<sup>0</sup> and 1min at 72C<sup>0</sup>,and final extention of 5 min at 72C<sup>0</sup> . 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<sup>0</sup>,followed by 35 cycles of 30s at 95C<sup>0</sup> ,1min at 56 C<sup>0</sup> and 1min at 72C<sup>0</sup>,and final extention of 5 min at 72C<sup>0</sup> . 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.

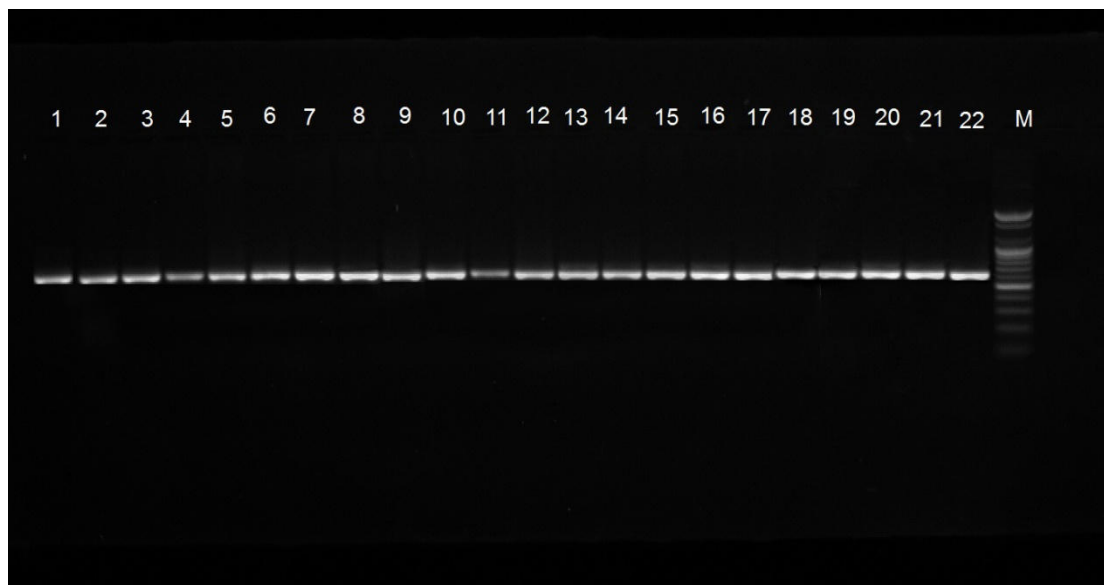


Figure (1): Agarose gel electrophoresis of PCR amplification products of *C.difficile* Toxin A (tcdA )gene .Expected products of amplification are 625bp.

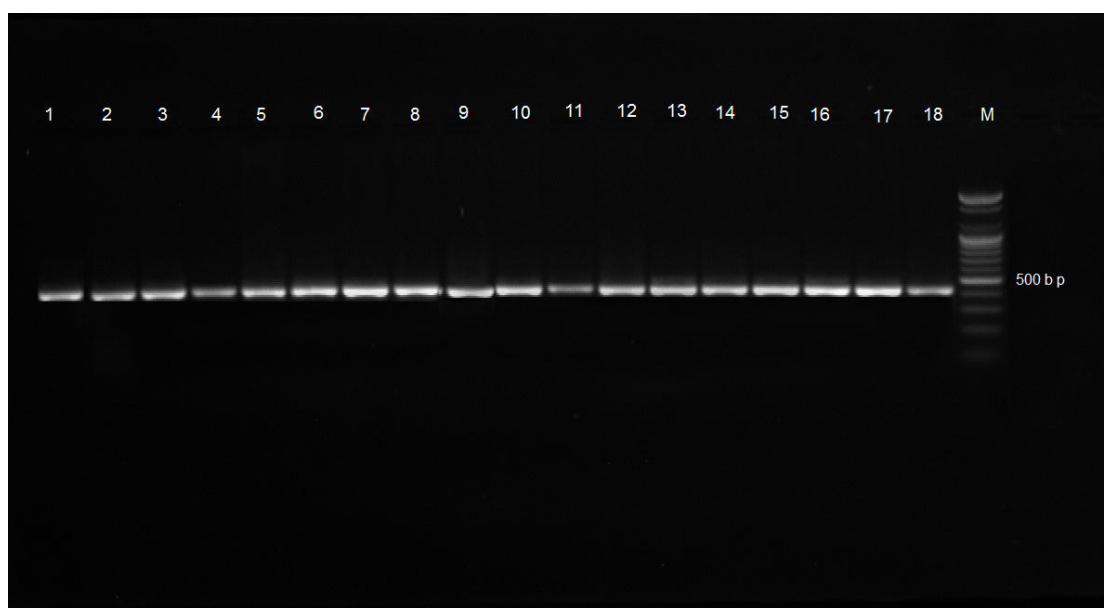


Figure (2): Agarose gel electrophoresis of PCR amplification products of *C.difficile* Toxin B (tcdB )gene .Expected products of amplification are 410bp.

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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