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ORIGINAL ARTICLE

PCR Versus Cytotoxicity Assay for Diagnosis of Antibiotics Associated Diarrhea Caused by *Clostridium difficile* infection

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ABSTRACT

Background: *Antibiotic Associated Diarrhea (AAD) is a common health problem in patients under antibiotic therapy.* ***Clostridium difficile*** *is the commonest cause of AAD.* ***Objectives****: our study aimed to compare between cytotoxicity assay and PCR for the diagnosis of Clostridium difficile antibiotic associated diarrhea as regard specificity and sensitivity.* ***Methodology:*** *Stool samples were collected from patients clinically suspected to have antibiotic associated diarrhea and proved by sigmoidscope from General Surgery Department in Tanta University. All samples were subjected to cytotoxicity assay to detect C-difficile toxin and PCR to detect ctd B gene encoding for toxin B.* ***Results:*** *Cytotoxicity test showed specificity 96% and sensitivity 90.5%.while PCR assay showed specificity 100% and sensitivity 98%.* ***Conclusion:*** *PCR is rapid, specific and sensitive in the diagnosis of C-difficile Antibiotic associated diarrhea.*

INTRODUCTION

Antibiotic Associated Diarrhea (AAD) is a common health problem. It occurs in 20% of patients who are under antibiotic therapy.[1-3] The severity and prognosis of AAD depend on the type of accused antibiotic. Laboratory diagnosis of AAD is a world challenge.[4] *Clostridium difficiIe* is the commonest causative agent of AAD. Pathogenesis is mainly via disturbance of normal gut microbiota or via toxin production that causes mucosal damage of GIT.[5-8]

AAD must be suspected in any patient receiving antibiotic within last 3 weeks.[9] *Clostridium difficile* produces 2 types of toxins which are toxin A and B. It has been proved that toxin B produces cytopathicity besides being cytotoxic.[10-12]

Toxin B is more effective than toxin A in destroying colonic epithelium.[13]

Clostridium difficile has been recently reclassified as *clostridioides difficile*.[14]

Different methods were developed for its diagnosis. Blood-enriched Cycloserine–Cefoxitin–Fructose agar were developed and are still used in the laboratory in order to select *C. difficile* colonies from enteric microbiota.[15-18] However, toxins can be tested by cytotoxicity assay, using a variety of cell lines with antitoxin neutralization to confirm the test. Although cytotoxicity assay is considered to be the gold standard test for diagnosis of *C-difficile* infection, it is time consuming and can not be used as a routine screening test. ELISA for glutamate dehydrogenase (GHD) antigen in stool is used for initial diagnosis. [19]

Diagnosis of *Clostridium difficile* in any patient having acute diarrhea > 3 time per 24 hours for at least 10 days is mandatory.[20]

Megacolon and hirschspurung disease are predisposing factors for *Clostridium difficile* superinfection.[21]

Clostridium difficile is gram positive anaerobe that is considered to be normal flora but its superinfection occurs due to disturbance of normal gut population or what is called gut microbiota by antibiotics especially clindamycin and flouroquinolones.[22]

Symptoms of AAD may include low grade fever, lower abdominal pain, watery to bloody diarrhea and septic shock in severe cases. Extracolonic presentation may occur in 5% of AAD. Sigmoidscope, rectoscope and colonoscope may show mild inflammation or severe colonic mucosal involvement in the form of pseudomembrane on gut mucosa.[23]

The Society for Healthcare Epidemiology of America (SHEA) 2017 recommended the use of one step or multistep tests to detect toxigenic *Clostridium difficile in* acute or chronic diarrhea with mucosal involvement .[24]

METHODOLOGY

Subjects and design

This study was carried out during the period from August2018 to August 2019. Thirty Patients were selected from General Surgery Department, Tanta University Hospital. They were suspected clinically to have antibiotic associated diarrhea and colitis.

Inclusion criteria:

Include watery, mucoid or bloody diarrhea that persist for more than 1 week after a history of prolonged antibiotic use as clindamycin or flourquinolones and mild fever with lower abdominal pain. All cases showed evident pseumembranous colitis during sigmoidoscope and/or colonoscope.

Exclusion criteria:

Was antibiotic use denying by the patient, recent antibiotic use in previous 24 h, recent barium enema during last 10 days, use of antacid or normal sigmoidscopic study. Thirty cases were included in the study. The age group ranged from 65-78years. Stool samples were collected in a clean leak proof containers and labeled with patient name and date of sample collection. The samples were transferred rapidly to the microbiological laboratory and processed. At least 2 ml of stool samples were used. All samples were subjected to cytotoxicity assay and PCR.

Ethical approval for this study was provided by ethical and research committee.

Cytotoxicity assay:

Cytotoxicity assay was performed using the *C-difficile* toxin antitoxin kit (Techlab, Blacksburg, va). Stool samples were washed by phosphate buffered saline and centrifuged. The deposit was discarded while the supernatant was added to 96 wells containing cell line of green African monkey .The test was considered positive if cytopathic rounding of cells occurs and prevented after neutralization with specific antitoxin to *Clostridium difficile*. The test was evaluated after 48hours.The test starts to show positive results after 24h while negative results were documented after 48h.

PCR Assay:

This assay aimed to detect the gene encoding for toxin B of *clostridium difficil*e (ctd B). This gene is 322 bp molecular weight. DNA was extracted from stool samples using Qiagene extraction mini stool kits. According to manufacturer's instructions. Four microliters of each extracted sample was added in 50ul reaction mixture to the primers sequence corresponding to forward and reverse primers respectively (table 1). The primer targets the *C-difficile* B toxin gene ctd B gene. Using thermal cycler 9600Perkin-Elemer and Taq polymerase (Qiagene), the amplification cycle were done consisting of initial denaturation step at 94^{0}C for 3 min., 30 cycles of 94 ^{0}C for 45 S then 56C for 45 S then 72^{0}C for 75S and final extension at 72^{0}C for 10 min. Amplicons were detected in 1.3% agarose gel stained by ethidium bromide by gel electrophoresis. Step ladder marker from 100 to 1000 was used. Positive control using pure DNA from toxogenic *C-difficile* strain ATCC9689 was used in lane 1. The target gene was detected at 322bp.

Table 1: Forward and reverse primers deigned for ctd B gene

Forward primer	(5′-TTTAGATACTACACACGAAG-3′)
Reverse primer	(5′-GCCATTATACCTATCTTAGC-3)′

RESULTS

This study was carried out on 30 patients suspected clinically as AAD and proven to have pseudomembranous colitis by sigmidscopy. Out of 30 samples 22 samples were positive by cytotoxicity assay while 8 samples were negative by cytotoxicity assay .By PCR all 22 samples were positive for gene encoding toxin B while 3 out of 8 cases that were negative by cytotoxicity test were positive by PCR.

Cytotoxicity test showed specificity 96% and sensitivity 90.5%. While PCR assay showed specificity 100% and sensitivity 98%. (table2)

Table 2: Shows specificity, sensitivity, positive predictive value and negative predictive value of cytotoxicity and PCR

Assay	Specificity	Sensitivity	PPV	NPP
Cytotoxicity	96	90.5	89.5	97.1
PCR	100	98	99	98.1

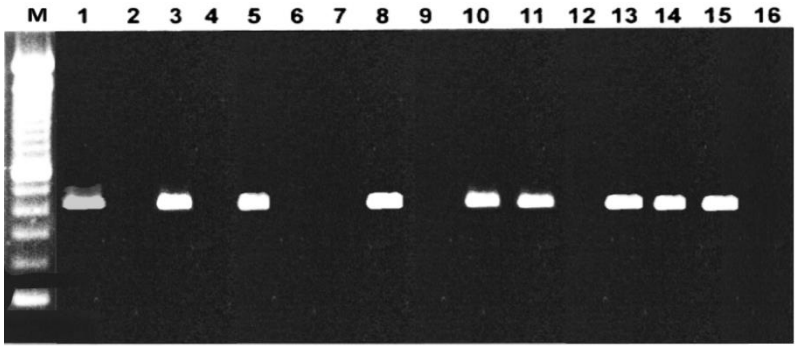

Photo1: Agarose gel electrophoresis for amplified product of ctdB gene

Lane 1: **Positive control** Lane 3**,** 5,8,10,11,13,14,15 **were positive**

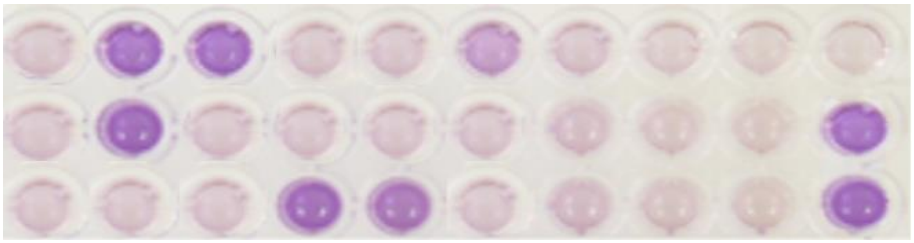

Photo 2: Cytotoxicity Assay test.
Dark color indicates negative results while light color indicates positive result

DISCUSSION

This study was carried out on 30 patients suspected clinically as AAD and proven to have pseudomembranous colitis by sigmidscopy. Out of 30 samples 22 samples were positive by cytotoxicity assay while 8 samples were negative. By PCR all 22 samples were positive for gene encoding toxin B (ctd B) while 3 out of 8 cases that were negative by cytotoxicity test were positive by PCR.

Cytotoxicity test showed specificity 96% and sensitivity 90.5% while PCR assay showed specificity 100% and sensitivity 98%.

This was in accordance with Deshpande et al[25] who found that PCR had a specificity of 96% and sensitivity of 95%.

This study was in accordance to Alcala et al[26] who found that the sensitivity, specificity, and positive and negative predictive values of the PCR assay were 93.5, 94.0, 73.0, and 98.8%, respectively.

Our study disagreed with Akerlund et al[27] who found that cytotoxicity assay had sensitivity, 90.9%, specificity, 95.2%, positive predictive value 70.2% and negative predictive value, 98.8%.

CONCLUSION

AAD is a common health problem. It needs urgent management to prevent mortality. *C-difficile* toxin is the key factor for the diagnosis. Although PCR assay is expensive but by measuring cost to benefit it must be used as a routine test for diagnosis of AAD. PCR is a rapid, specific and sensitive method.

Conflicts of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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