PCR Versus Toxigenic Culture in Diagnosis of Antibiotic-Associated Diarrhea Due to Clostridium Difficile Infection

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ABSTRACT
Background: Clostridium difficile infection (CDI) is a leading cause of healthcare-associated infections accounting for significant disease burden and mortality. The clinical spectrum of C difficile ranges from asymptomatic colonization to toxic megacolon and fulminant colitis. **Objective:** The present study aimed to evaluate the sensitivity and specificity of PCR in comparison with toxigenic culture for diagnosis of antibiotic-associated diarrhea due to Cl difficile infection. **Materials and methods:** This comparative study was conducted on 80 patients with antibiotic associated diarrhea (AAD). Toxigenic culture (TC) was done for detection of toxigenic Cl difficile and, PCR assay was done for detection of tcdA and tcdB genes and results of both methods were compared. **Results:** Out of 80 diarrhea patients included in the study, 12 (15%) were positive and 68 (85%) were negative for toxigenic culture. Out of 80 diarrhea patients included in the study, 32 (40%) were positive and 48 (60%) were negative for PCR. The sensitivity, specificity, PPV and NPV of PCR in diagnosis of Cl difficile infection were 100%, 70%, 61.5% and 100% respectively. Also, there was a highly significant difference between positive and negative results as detected by PCR. **Conclusion:** we can conclude that PCR is a highly sensitive method (100% sensitivity) as compared to TC in diagnosis of antibiotic-associated diarrhea due Cl difficile infection.
Keywords: Toxigenic culture, PCR, Clostridium difficile.

INTRODUCTION

Clostridium difficile is an important cause of nosocomial infections, it is recognized as the major cause of healthcare antibiotic-associated diarrhea. Antibiotics used for treating every kind of infection may potentially promote C. difficile infection (CDI). After antibiotic therapy, the protective intestinal microbiota is disrupted allowing ingested or resident C. difficile to colonize the gastrointestinal tract and infect the host. Antibiotic resistance enables C. difficile to grow in the presence of drugs, so strains resistant to multiple agents may have a selective advantage for their diffusion from the usage of these antibiotics [1].

Symptoms of C. difficile infection (CDI) range from asymptomatic carriage to mild diarrhea, colitis, severe life threatening pseudomembranous colitis and to fulminant colitis [2]. In the past several years, a rapid increase in the incidence of C difficile infection (CDI) has occurred with recognition of new, highly virulent strains causing global outbreaks [3]. Rapid and accurate diagnosis of CDI is essential both for improving outcomes of patients with CDI and for reducing horizontal transmission in health care facilities [4].

C. difficile expresses two major virulence factors, which are toxin A (enterotoxin) and toxin B (cytotoxin) encoded via tcdA and tcdB genes respectively. As well as inflammation and fluid secretion, Clostridial exotoxins bind to the human intestinal cells and are responsible for the damage to the intestinal mucosa. Various types of virulence factors contribute to the pathogenicity of C. difficile within the gastrointestinal tract [5, 6].

There are various tests for diagnosis of CDI in laboratories. Some of these tests are enzyme immunoassay (EIA), glutamate dehydrogenase (GDH),

cytotoxicity assay (CA), toxigenic culture and PCR. Enzyme immunoassay is a rapid method and is done directly on stool samples. Although this test is very fast, it has very low sensitivity. The GDH test detects glutamate dehydrogenase enzyme in the cell wall of C. difficile [7]. Toxigenic culture (TC) is considered as the reference method to detect toxigenic C. difficile and remains a reference standard for evaluating new molecular methods. Although the turnaround time of this method is too long for routine diagnosis (2–5 days), culture is essential for subsequent typing, molecular analysis and determination of antimicrobial susceptibility [8].

The symptoms of the related infection depend on toxin-encoding pathogenicity locus (PaLoc) in the bacterial genome. The PaLoc is a conserved and stable genetic unit, which is 19.6 kb and contains the tcdA and tcdB genes. Other PaLoc genes are tcdR and tcdC, which encode positive and negative regulators for tcdA and tcdB toxins. The PaLoc is located at the same site on the chromosome and includes three additional tcd open reading frames (ORFs); tcdD, tcdE, and tcdC, and ORFs for the insertion sequences cdd-2, cdd-3 that is located upstream and downstream of the PaLoc, present in both non-toxigenic and toxigenic strains[9-10].

Studies on C. difficile indicated that it has the ability to ferment low molecular weight substrates of amino acids. Glutamate has the main dependence of the other amino acid metabolism. Glutamate dehydrogenase (GDH) is a constructor enzyme produced in large amounts by all strains of C. difficile independent of their toxigenic or non-toxigenic forms [11, 12]. Detection of this enzyme by immunoassay methods has been considered a valid technique with proper sensitivity for the screening of C. difficile in

stool samples [13]. More comprehensive diagnostic assays for C. difficile infection are based on clinical symptoms in combination with laboratory tests for screening C. difficile toxins and GDH. GDH enzyme detection methods do not distinguish between toxigenic and non-toxigenic strains, thus a toxin assay is required while a definitive diagnosis is necessary [14]. In addition, approaching toxin is essential for epidemiological research, optimal management, and prevention programs. Molecular methods for the diagnosis of C. difficile infection have been studied far less than those used to diagnose other infectious diseases. Various nucleic acid amplification tests are commonly used for detection of toxigenic C. difficile [15].

The aim of the present study was to evaluate the sensitivity and specificity of PCR in comparison with toxigenic culture for diagnosis of antibiotic-associated diarrhea due Cl difficile infection.

MATERIALS AND METHODS

This comparative study was conducted at Microbiology and Immunology Department, Faculty of Medicine, Benha University Hospital from August 2021 to December 2021 on 80 hospitalized patients, with (AAD) antibiotic associated diarrhea. Inclusion criteria were: (loose stools more than 3 times per day for at least 48 h and antibiotic use for at least 48 h within 2 weeks prior to the onset of diarrhea). exclusion criteria were: (patients diagnosed with inflammatory bowel disease, HIV, amoebiasis, and patients used laxative 48h prior to the onset of diarrhea , were all excluded from the study).

Stool specimens were collected from from 80 patients with AAD admitted in different hospital wards, and immediately transferred to the microbiology laboratory. About one gram of stool specimen was suspended in a tube containing one mL of BHI broth, and one mL of ethanol 96% (ethanol shock) for 45 min. Then this suspension was cultured on CCFA (cycloserine - cefoxitin fructose agar; under anaerobic condition at 37°C for 72 h). The colonies which contained Gram-positive bacilli with 1-3 mm diameter, white to gray color, and horse odour were regarded as C. difficile. The isolated strains were stocked in BHI broth containing 15% glycerol and tored at -20°C.

Toxigenic culture was performed for isolated C. difficile strains. Vero cells were grown in a flask containing Dulbecco's modified Eagle's medium, 100 U/ml penicillin-streptomycin and 10% fetal bovine serum (FBS), and incubated at 37°C and 5% CO_2 for 3-5 days. The cells were trypsinized and counted. About 10,000 cells were added to the wells of microtiterplate and were incubated at 37°C and 5% CO_2 for 24 h to reach about 80% confluency. C. difficile strains were cultured in BHI broth for 5-7 days at 37°C, then the culture medium was centrifuged (10 min at 1500 g). The obtained supernatants were filtered (0.22 μm pore size), and 200 μl of filtrate were added to Vero cell culture (microtiter plate). The microtiter plate was incubated

for 24 - 48 h at 37°C and 5% CO_2. C. difficile strains, which produce toxin (positive result), cause cytopathic effects in more than 50% of the cell monolayer. Supernatant obtained from a toxigenic C. difficile strain, which was previously isolated from a diarrhea patient, was used as a positive control in toxigenic culture test [16].

Identification of tcdA and tcdB genes by PCR:

A single colony from every isolate was used for DNA extraction as described by the manufacturer (Thermo Scientific), primers used in assay listed in table (1). For each gene, the PCR was run in 20 μl reaction mixture containing 10 μl master mix PCR, 2 μl DNA template, 20 pmol of each primer and 6.4 μl PCR grade water. PCR was performed in a thermocycler using the following conditions: 5 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The presence of each gene was determined by electrophoresis on a 1.5% agarose gel. In each PCR run, DNA template from a toxigenic C. difficile and water were used as positive and negative controls, respectively.

Table (1): Primers used in this study

Genes	Primer	Sequence	Amplicon
tcdA	tcdA-F3345	GCATGATAAGGCAACTTCAGTGGTA	629
	tcdA-R3969	AGTTCCTCCTGCTCCATCAAATG	
tcdB	tcdB-R6079A	GCATTTCTCCATTCTCAGCAAAGTA	410
	tcdB-F5670	CCAAARTGGAGTGTTACAAACAGGTG	

Ethical consent:

An approval of the study was obtained from Benha University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

All statistical analyses were carried out in STATA/SE version 11.0 for Windows. Mean ± SD was used to describe the quantitative data and proportion was used to describe the qualitative data. The test of proportion (z test) was used to compare the results of the PCR with the results of the reference test (TC) and a P-value ≤ 0.05 was considered statistically significant. The sensitivity and specificity, were calculated for PCR against the reference test and receiver operating characteristic (ROC) curve analysis was carried out. The sensitivity of a test is the probability that the test is positive given a patient has the condition. Sensitivity = Probability [test (T+) | diseased (D+)]. The specificity of a test is the probability that the test is negative given

a patient does not have the condition. Specificity = Probability [test (T-) | diseased (D-)]. Positive predictive value is the probability that a patient with abnormal test results is truly abnormal. PV+ = Probability [diseased (D+) | test (T+)]. Negative predictive value is the probability that a patient with normal test results is truly normal. PV- = Probability [diseased (D-) | test (T-)]. P ≤ 0.05 was considered significant.

RESULTS

Considering toxgenic culture as a reference method for diagnosis of Cl difficile infection, the results of the detection of tcdA and tcdB genes by PCR regarding specificity and sensitivity were evaluated in relation to toxgenic culture results. Out of 80 patients with AAD included in the study, 12 (15%) were positive and 68 (85%) were negative for toxigenic culture as shown in figure (1).
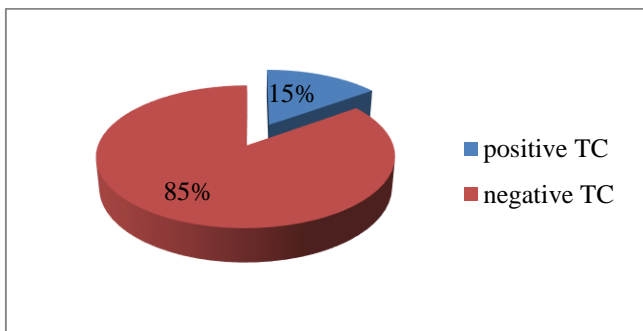

Figure (1): Distribution of studied patients regarding toxigenic culture results for detection of Cl difficile infection

Out of 80 patients with AAD included in the study, 32(40%) were positive and 48 (60%) were negative for PCR as shown in figure (2).
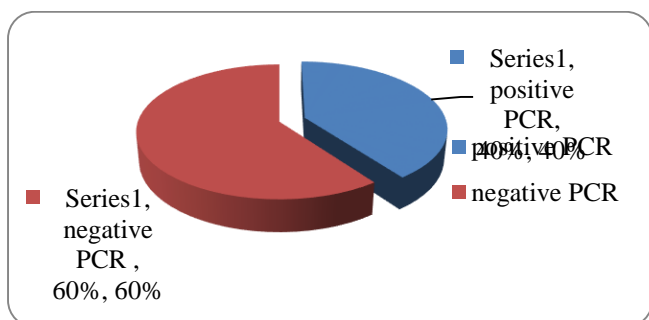

Figure (2): Distribution of studied patients regarding PCR results for detection of Cl difficile infection

The results of detection of tcdA and tcdB genes by PCR regarding specificity and sensitivity were evaluated in relation to toxigenic culture results. The result of PCR revealed that, all positive cases for toxigenic culture, were positive for PCR patient, and out of 68 negative culture cases, 48 cases only gave negative PCR result and 20 patients were positive for PCR despite of negative culture. Study revealed that,

the sensitivity, specificity, PPV and NPV of PCR in diagnosis of Cl difficile infection were 100 %, 70.5 %, 61.5% and 100 % respectively. Also, there was a highly significant difference between positive and negative results as detected by PCR as shown in table (2). Low specificity of PCR method means it cannot distinguish between colonization and infection this gave a chance of overdiagnosis and increase the risk of multidrug-resistant pathogens.

Table (2): Comparison between results of PCR and toxigenic culture in diagnosis of antibiotic-associated diarrhea due to Clostridium difficile infection

Toxigenic culture PCR	Positive		Negative		p-value
	No.	%	No.	%	
Positive	12	100%	20	29.5%	<0.001
Negative	0	0%	48	70.5%	
Total	12	100.0%	68	100.0%	

Sensitivity= 100%. Specificity=70.5%. Positive predictive value (PPV) = 61.5%, Negative predictive value (NPV) =100% P value <0.001 (highly significant).

Receiver operating characteristic (ROC) curve analysis was carried out for PCR in comparison with TC and revealed that, PCR is a good test in diagnosis of antibiotic-associated diarrhea due to Clostridium difficile infection as shown in figure (3).
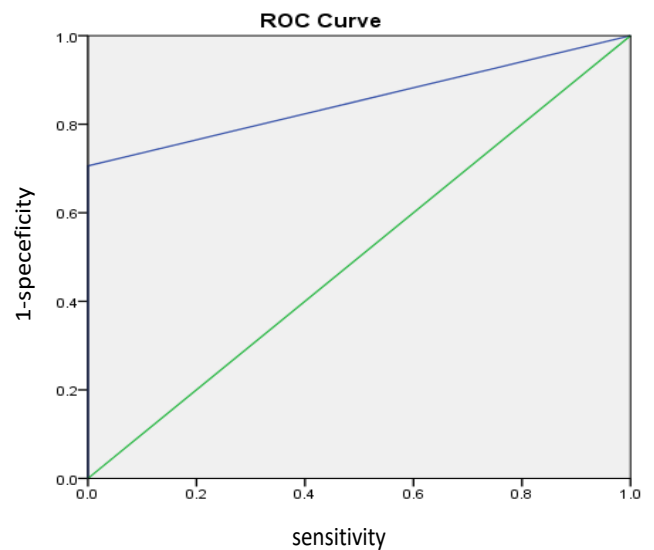

Figure (3): Receiver operating characteristic (ROC) curve for PCR for in comparison to TC in diagnosis of antibiotic associated diarrhea due to Clostridium difficile infection.

DISCUSSION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium, and the leading cause of health-care associated infective diarrhea. Risk factors for the disease include previous hospitalizations, advanced age and the use of antibiotics [17]. C difficile spores play an important role in disease transmission. Spores can persist in the environment for up to several

months and are resistant to stresses such as heat, oxygen, and exposure to routine disinfectants [18].

Out of 80 diarrhea patients representing the study group, 12 (15%) patients were positive for Cl difficile infection as detected by TC. This percentage is near that of **Mirzaei et al.** [19] who conducted a study on one hundred stool specimens were taken from diarrheal patients in hospitals of Babol. Also, **Barbut et al.** [20] who studied 285 fresh stools from 285 patients in Paris with suspected Cl difficile associated diarrhea CDAD.

This study revealed that the sensitivity and specificity of detection of tcdA and tcdB genes by PCR assay were 100% and 70.5% respectively in comparison with TC results. Low specificity of PCR method means it cannot distinguish between colonization and infection this give a chance of overdiagnosis and increase the risk of multidrug- resistant pathogens. Our results come in accordance with **Le Guern et al.** [21] who analyzed the different options available for a molecular diagnosis of C. difficile infection. They reported a high sensitivity and rapidity and low specificity of PCR assay and that additional efforts should focus on the discrimination between infection and colonization. Reporting the DNA load of toxigenic C. difficile in the stool sample may represent a solution. Diagnostic algorithms combining immunoassays and PCR assay could also improve the specificity and reduce the global cost of this analysis. **Ylisiurua et al.** [22] who evaluated 3 methods for detecting CD toxins: enzyme immunoassay (EIA), loop-mediated isothermal amplification (LAMP) assay and PCR assay, as direct identification methods from stool specimens considering toxigenic culture (TC) as the reference method. They recorded a similar results regarding PCR sensitivity. But, on the other hand they reported a high specificity (100%) of PCR assay in diagnosis of Cl difficile infection, which contradict our results that recorded 70.5% specificity of PCR assay. **Jamal et al.** [23] evaluated the performance of the GeneExpert C. difficile PCR assay for the detection of toxins from fecal specimens and cooked meat broth culture using toxigenic stool culture as reference method, for the diagnosis of C. difficile infection (CDI) in a community setting. They contradict our results as sensitivity was 81.3 % while specificity was 100%. **Xiao et al.** [24] who performed a study to evaluate the clinical application of three methods for detecting Clostridium difficile in fecal samples: (1) the toxigenic culture, as reference method, (2) the VIDAS enzyme immunoassay (EIA): the VIDAS glutamate dehydrogenase (GDH) assay and toxin A/B assay were used to detect GDH antigen and A/B toxin, and (3) the GeneXpert PCR assay. They recorded results of GeneXpert C. difficile PCR assay similar to our study regarding sensitivity, and NPV (100% and 100% respectively), but a contradictory results regarding specificity and PPV (96.8% and 88.9 % respectively). On the other hand, **Tenover et al.** [25] assessed the performance of the C. difficile PCR assay on stool specimens collected from patients suspected of having Clostridium difficile infection (CDI). Compared to results for toxigenic culture, the sensitivity, specificity, and positive and negative predictive values of the PCR assay were 93.5 %, 94.0 % 73.0 % and 98.8%, respectively. These results were near to our results regarding PPV and NPV, but disagree with our results regarding sensitivity and specificity.

CONCLUSION

From this study we can could conclude that PCR for detection of tcdA and tcdB genes is highly sensitive method (100% sensitivity) compared to TC in diagnosis of antibiotic-associated diarrhea due Cl difficile infection. But it has a 70.5% specificity thus cannot distinguish between colonization and infection this give a chance of overdiagnosis and increase the risk of multidrug-resistant pathogens.We recommend utilizing a multistep testing algorithm to maximize the sensitivity and specificity of available C. difficile tests and avoid the diagnosis of asymptomatic colonizers.

REFERENCES
1. **Spigaglia P (2016):** Recent advances in the understanding of antibiotic resistance in Clostridium difficile infection. Ther Adv Infect Dis., 3 (1): 23-42.
2. **De Boer E, Zwartkruis-Nahuis A, Heuvelink A et al. (2011):** Prevalence of Clostridium difficile in retailed meat in the Netherlands. Int J Food Microbiol., 144: 561-564.
3. **Warny M, Pepin J, Fang A et al. (2005):** Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. Lancet, 366 (9491): 1079-1084.
4. **Guerrero D, Chou C, Jury L et al. (2011):** Clinical and infection control implications of Clostridium difficile infection with negative enzyme immunoassay for toxin. Clin Infect Dis., 53 (3): 287-290.
5. **Orrell K, Zhang Z, Sugiman-Marangos S et al. (2017):** Clostridium difficile toxins A and B: Receptors, pores, and translocation into cells. Crit Rev Biochem Mol Biol., 52: 461-73.
6. **Chandrasekaran R, Lacy D (2017):** The role of toxins in Clostridium difficile infection. FEMS Microbiol Rev., 41: 723-50.
7. **Carman R, Wickham K, Chen L et al. (2012):** Glutamate dehydrogenase is highly conserved among Clostridium difficile ribotypes. J Clin Microbiol., 50: 1425-1426.
8. **Balamurugan R, Balaji V, Ramakrishna B (2008):** Estimation of faecal carriage of Clostridium difficile in patients with ulcerative colitis using real time

polymerase chain reaction. Indian J Med Res., 127: 472-477.

9. **Martin-Verstraete I, Peltier J, Dupuy B (2016):** The Regulatory Networks That Control Clostridium difficile Toxin Synthesis. Toxins (Basel), 8: 153-158.

10. **Brouwer M, Mullany P, Allan E *et al.* (2016):** Investigating Transfer of Large Chromosomal Regions Containing the Pathogenicity Locus Between Clostridium difficile Strains. Methods Mol Biol., 1476: 215-22.

11. **Girinathan B, Braun S, Sirigireddy A *et al.* (2016):** Importance of Glutamate Dehydrogenase (GDH) in Clostridium difficile Colonization In-Vivo. PloS One, 11:e0160107.doi:10.1371/ journal.pone.0160107.

12. **Girinathan B, Braun S, Govind R (2014):** Clostridium difficile glutamate dehydrogenase is a secreted enzyme that confers resistance to H2O2. Microbiology, 160: 47-55.

13. **Yoldas O, Altindis M, Cufali D *et al.* (2016):** A Diagnostic Algorithm for the Detection of Clostridium difficile-Associated Diarrhea. Balkan Med J., 33: 80-6.

14. **Moon H, Kim H, Hur M *et al.* (2016):** Comparison of Diagnostic Algorithms for Detecting Toxigenic Clostridium difficile in Routine Practice at a Tertiary Referral Hospital in Korea. PloS One, 11: e0161139. doi:10.1371/journal.pone.0161139.

15. **Martinez-Melendez A, Camacho-Ortiz A, Morfin-Otero R *et al.* (2016):** Current knowledge on the laboratory diagnosis of Clostridium difficile infection. World J Gastroenteral., 23: 1552-67.

16. **Pancholi P, Kelly C, Raczkowski M *et al.* (2012):** Detection of toxigenic Clostridium difficile: comparison of the cell culture neutralization, Xpert C. difficile, Xpert C. difficile/Epi, and Illumigene C. difficile assays. J Clin Microbiol., 50: 1331-1335.

17. **Smits W, Lyras D, Lacy D *et al.* (2016):** Clostridium difficile infection. Nat Rev Dis Primer., 2: 16020. https://doi.org/10.1038/nrdp.2016.20 PMID: 27158839.

18. **Sethi A, Al-Nassir W, Nerandzic M *et al.* (2010):** Persistence of Skin Contamination and environmental shedding of Clostridium difficile during and after treatment of C. difficile infection. Infect Control Hosp Epidemiol., 31: 21-27.

19. **Mirzaei E, Rajabnia M, Sadeghi F *et al.* (2018):** Diagnosis of Clostridium difficile infection by toxigenic culture and PCR assay, Iran. J Microbiol., 10: 287-293.

20. **Barbut F, Kajzer C, Planas N *et al.* (1993):** Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of clostridium difficile-associated diarrhea. Journal of Clinical Microbiology, 31: 963-967.

21. **Le Guern R, Herwegh S, Courcol R *et al.* (2014):** Molecular methods in the diagnosis of Clostridium difficile infections: an update. Expert Review of Molecular Diagnostics, 13: 681-92.

22. **Ylisiurua P, Koskela M, Tuokko H (2013):** Comparison of antigen and two molecular methods for the detection of clostridium difficile toxins. Scand J Infect Dis., 45: 19-25.

23. **Jamal W, Pauline E, Rotimi V (2014):** Comparative performance of the GeneXpert C. difficile PCR assay and C. diff Quik Chek Complete kit assay for detection of Clostridium difficile antigen and toxins in symptomatic community-onset infections. Int J Infect Dis., 29: 244- 8.

24. **Xiao Y, Liu Y, Qin X (2020):** Comparative Study of Clostridium difficile Clinical Detection Methods in Patients with Diarrhoea. Can J Infect Dis Med Microbiol., 20: 8753284. doi: 10.1155/2020/8753284

25. **Tenover F, Novak-Weekely S, Persing D (2010):** Impact of strain type on detection of toxigenic clostridium difficile: comparison of molecular diagnostic and enzyme immunoassay approaches. Journal of clinical Microbiology, 48 (10): 3719-24.