Detection of nosocomial toxigenic Clostridium difficile associated diarrhea in children by conventional PCR

Hala S Arif (MBChB/CABP)[1] , **Jabbar Salman Hassan** (PhD) [2], **Thana R Abdul Rhman**(PhD)[3] **and Wurood J Hassan**(MBChB)[4]

Abstract

Background: *Clostridium difficile* is a gram positive anaerobic spore forming bacteria. C. difficile–associated disease is a critical clinical issue that is accepted to happen mainly after hospitalization and used of expansive range anti-infection agents.

Objective: To define the rate of *C. difficile* infections isolated from children patients suffering from diarrhea, detection profile toxigenicity of C. difficile strains for toxin A and toxin B by using of PCR, and revise different risk factors of *C. difficile* infections.

Patients and Methods: This cross-sectional study included 50 patients who hospitalized for at least 2 days before the appearance of three or more unformed or liquid stools for 24h, genomic DNA was extracted by using 10% fecal supernatant and a ready kit was used for extraction according to manufacturer instructions. Molecular detection of toxigenic *C. difficile* done by using the specific primer sequences in polymerase chain reaction.

Results: Current study showed diarrhea was the most prominent complain among the study population accounting for 41(82%), of whom 39(78%) presented with watery diarrhea. 38(76%) patients had no fever. The most comorbid disease was inflammatory bowel disease (IBD) with 7 (14%) patients. Forty-six (92%) cases had no history of hospitalization in the last 3 months versus only 8% had such history. PCR revealed that 16 (32%) samples were positive for tcdB gene, while all samples were negative for genes tcdA.

Conclusion: The study showed a relationship between previously diagnosed patients with IBD and exacerbations with *C. difficile* infections (CDIs). Clinically the toxin B alone elicits severe enterotoxic effects which increase rate of (CDIs).

Key words: Inflammatory bowel disease, Clostridium difficile infections, Clostridium difficile–associated disease, toxin A and toxin B.

Corresponding Author: jabbarsalman30@yahoo.com

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[1] Ministry of health- AL- Imammian AL- Kadhmain City Teaching Hospital- Baghdad- Iraq.
[2,3] Medical Microbiology department - College of Medicine- Al-Nahrain University -Iraq.
[4]Ministry of health- AL- Imammian AL- Kadhmain City Teaching Hospital- Baghdad-Iraq.

Introduction

Antibiotic-associated diarrhea and pseudomembranous colitis is the most common complications of Clostridium difficile and this bacteria considers as a reason for hospital acquired diarrhea [1] C. difficile is a gram positive anaerobic with ability to form spore, C. difficile–associated disease (CDAD) is a critical clinical issue that is accepted to happen mainly after hospitalization and used of expansive range anti-infection agents, it particularly affects the old age and children [2] there are toxin producing and non-producing toxin C. difficile strains. Toxigenic C. difficile, is

establish in digestive system of hygienic individual as a segment of typical microbiota , isn't a wellbeing hazard [3] yet in individual with risk factors (antimicrobial use, older age patients, long-period hospitalization)this bacteria may be lead to diarrhea of various severities, sometime reach to life-threatening pseudomembranous colitis [4] there has been an even higher increase in incidence of CDI among patients with inflammatory bowel disease (IBD) [5] There are some typical IBD-related risk factors that stimulate C. difficile infection (CDI) such as prolong use of steroid therapy. Thus, in many occasions, IBDs cannot be easily distinguished from CDI (6). It is well known that these bacteria possess many virulence factors that enhance their pathogenicity. Of these, exotoxins are considered the most important ones. C. difficile synthesis many types of toxins; the major two of which are toxin A and toxin B. However, other toxin was described recently as a binary toxin [7] The action of toxin A, toxin B lead to perturbation of the actin cytoskeleton causing and disturbance of closely junctions epithelial cells of gastrointestinal tract, the two-toxin act as cytotoxic and proinflammatory with lead to liquid aggregation and broad harm to the digestive organ [8].

Also, cytotoxic effect of these toxin stimulates macrophage and other immune cell such as mast cell to liberation inflammatory cytokines prompting further liquid accumulation which causes intestinal inflammation. It is interesting that the effect of toxin B is not limited to damaging the intestine, but rather to it is cardiotoxic and can cause bleeding in the lungs [9]. Some new investigation has shown that C. difficile that deliver toxin B however not toxin A can cause serious gut infection mainly diarrhea in humans [10]. Toxins A and B are encoded by the genes tcdA and tcdB, which are situated on the pathogenicity island PaLoc, which likewise incorporates negative and positive regulators tcdC and tcdR [11].

This study was aimed to define the rate of *C. difficile* infections isolated from children patients suffering from diarrhea, detection profile toxigenicity of *C. difficile* strains for toxin A and toxin B by using of PCR, and revise different risk factors of *C. difficile* infections.

Patients and Methods

A cross sectional study was conducted in AL-Imamein AL-Kadhimein Medical City, fifty patients aged between (few days to more than 5 years) from Baghdad and its suburbs were enrolled in this study. The study was conducted through the period from November 2015 to end of April 2016.

The choice of patients depended on following criteria: presence no less than three unformed or liquid stools during 24h for no less than 2 days, Hospitalization at least 48h preceding the presence of unformed or liquid stool. (With exception to patients with inflammatory bowel disease who already presented with diarrhea and have not recently been hospitalized). The control group consisted of 30 children who came for routine checkup for general stool test,

without signs and symptoms of intestinal tract infections, and did not receive antibiotic.

The stool samples were separated into two parts, the first one (0.5 mg) was set up for wet smear preparation, examination by light microscope for detection of protozoa, helminth and other intestinal tract infections and the second portion used for DNA extracted. Genomic DNA was extracted by using 10% fecal supernatant from patients and control group using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) following guidelines of the manufacture.

Molecular detection of Toxigenic Clostridium difficile: Toxigenic C. difficile detection was performed by using the special primer sequences in conventional PCR to recognize the presence of Toxins A and B gene these primers were selected according to Terhes et al [12]; Cohen et al [13] and housekeeping gen primers which is in this study was Triose phosphate isomerase (tpi) prepared depend on Lemee et al [14] used as an experimental internal control during protocols of PCR a positive control for confirming the acceptability of the extracted DNA to template, those genes synthesized in Alpha DNA® (Canada) shown in table (1). The primers were diluted by adding nuclease free water according to the manufacturer guidelines. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet under septic conditions to avoid contamination. For each reaction within single pre-mixed PCR reaction tube, 2µl from forward primer and reverse primer for tcdA gene encoded toxin A were added. Five microliter of DNA template was added for each reaction tube. Twelve and a half microliters of GoTaq® Green Master Mix was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease –Free and tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. The same reaction was repeated for tcdB gene encoded toxin B.

PCR mixture without DNA template (non-template negative control) were utilized as negative control. The tubes were put on the PCR machine (Cleaver Scientific Thermal Cycler TC32/80).The PCR program, with the right cycling conditions pre-installed, amplification for Toxigenic C. difficile genes was as follows: 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec, terminating in 72°C for 7 min, 10 µl of each PCR product was subjected to 1% (wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 µg /ml; Sigma) Five microliters of the 100bp DNA ladder was subjected to electrophoresis in a single lane. Served as marker during PCR products electrophoresis Amplicon representation was performed using an UV light transilluminator.

Table (1): Primer sequences and gene targets for C. difficile with Triose phosphate isomerase (tpi).

		Nucleotide sequences	Reference	Products
Genes		**3') ►---------- (5'**		**bP**
tcdA	F	CCCAATAGAAGATTCAATATTAAGCTT	[12]	
	R	GGAAGAAAAGAACTTCTGGCTCACTCA GGT		251
tcdB	F	GGTGGAGCTGCTTCATTGGAGAG	[13]	
	R	GTGTAACCTACTTTCATAACACCA		399
Tpi	F R	AAAGAAGCTACTAAGGGTACAAA CATAATATTGGGTCTATTCCTAC	[14]	230

Statistical Analysis

Statistical Analysis system (SAS) software was used for all statistical analysis continuous variables were expressed in mean ± standard deviation (SD). The Pearson's Chi-square test or Fisher exact test was used for comparing the categorical variable. A two-sided significant level of 0.05 was considered to indicate a statistically significant difference

Results

Demographic information of the patients: The characteristics of the study population are shown in table (2). In the current study 16 patients proved as infected with C. difficile by PCR, there was slightly higher in bottle feeding than breast feeding also regarding gender girls 9 (40.9%) had the higher prevalence of C. difficile than boys 7 (25%) but this differences not statistically significant for the distributions of C. difficile based on age groups, gender and type of feeding.

Table (2): Relationship between clostridia infection and patient characteristics.

Patient characteristics		Positive PCR	Negative PCR	P value
Age group	≤ 28 days (3 cases)	1(33.3%)	2 (66.7%)	0.281^{NS}
	>28 days- less than 1 year (21 cases)	7 (33.3%)	14(66.7%)	
	1-5 years (14 cases)	2 (14.3%)	12 (85.7%)	
	>5 years (12 cases)	6 (50%)	6(50%)	
Gender	boys	7 25%	21 75%	0.231^{NS}
	girls	9 40.9%	13 59.1%	

Residence	Urban	14 31.1%	31 68.9%	0.686^{NS}
	Rural	2 40%	3 60%	
Type of feeding	Breast feeding	0 0%	3 100%	0.157^{NS}
	Bottle feeding	5 50%	5 50%	
	Mixed feeding	3 21.4%	11 78.6%	

* Statistical significance p<0.05, NS= non-significant association.

Results revealed that acute diarrhea is the most prominent complain in 41 (82%). Thirty-nine (78%) presented with watery diarrhea, 38 (76%) from patients have no fever, the most comorbid diseases in study group were inflammatory bowel disease (IBD) 7 (14%), forty-six (92%) of cases had no history of hospitalization in the last 3 months, only 8% had history of hospitalization in the last 3 months. (but most of them were admission to the hospital during this diarrheal attack) table (3).

Table (3): Distribution of patients in relation to the different clinical picture.

Clinical features		**No.**	**%**
Duration of diarrhea	Acute diarrhea	41	82
	Chronic diarrhea	9	18
Type of diarrhea	Watery	39	78
	bloody	11	22
Presence of fever	Yes	12	24
	No	38	76
Comorbid diseases	IBD	7	14
	Nephrotic syndrome	1	2
	Chest infection	1	2
	ALL	1	2
	No disease	40	80
History of hospitalization in the last 3 months	Yes	4	8
	No	46	92

Molecular detection: Conventional PCR was done for the amplification of tcdA gene encoded toxin A and tcdB gene encoded toxin B by using specific set of primers sequences. The results showed that, amplification products were

obtained for tcdB gene in 16 (32%) out of 50 stool samples PCR product of this gene was 399 bp. Figure (1). No amplification products were obtained with tcdA gene. Regarding control group all control groups were negative for toxigenic *C. difficile.*

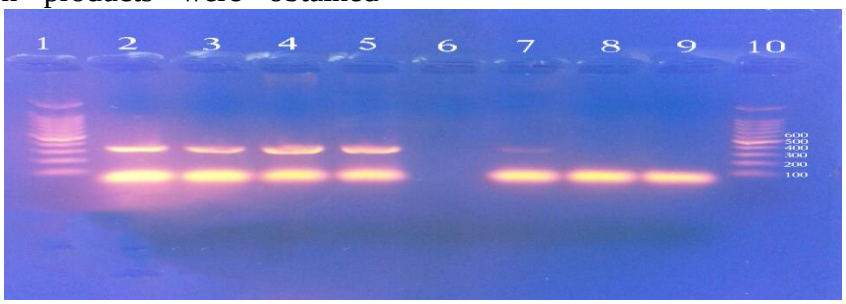

Figure (1): Gel electrophoresis (1% agarose,7v/cm2,1.5hrs) of the PCR products, lane1 and 10(MW): One hundred base pairs DNA ladder; lane (2-5& 7): Positive sample for toxin B (tcdB gene 399bp); lane 8 & 9: Negative sample; lane 6: Negative control.

Relationship between C. difficile infection and clinical feature of patients:

Out of 16 cases were PCR positive for C. difficile, 10 (25.6%) presented with watery diarrhea 6 (54.5%) patients complaining of bloody diarrhea, most presentation was acute diarrhea 11 (26.8%) followed by chronic diarrhea 5 (55.6%), inflammatory bowel disease (IBD) was the most comorbid disease 6 (60%), fifteen (32.6%) of positive cases had no history of hospitalization in the last 3 months Table (4).

Table (4): Distribution of patients with clinical feature and inflammatory bowel disease (IBD).

Clinical feature		Positive PCR	Negative PCR	P value
Type of diarrhea	Watery	10 (25.6%)	29 (74.4%)	0.070^{NS}
	Bloody	6 (54.5%)	5 (45.5%)	
Duration of diarrhea	Acute	11 (26.8%)	30 (73.2%)	0.103^{NS}
	Chronic	5(55.6%)	4(44.4%)	
inflammatory bowel disease (IBD)	Yes	6 (60%)	4 (40%)	0.034^{*}

	No	10 (25%)	30 (75%)	
History of hospitalization in the last 3 months	Yes	1 (25%)	3 75%	0.754^{NS}
	No	15 (32.6%)	31(67.4%)	

Relationship between antibiotics and *C. difficile* infection:

Nine antibiotics were used by patients, all patients received more than one antibiotic, the treatment periods were different according to the type of infection, the mean duration of treatment was 8.9 □ 12.3 days, minimum duration was 2 days and maximum duration was 60 days. Majority of the patients had been treated by Cefotaxime 37(74%) and least of them treated by Meropnem and Amikacins 2 (4%). Regarding positive PCR result revealed that Cefotaxime, Meropenem and Imuran(azathioprine) were associated with infection caused by *C. difficile* table (5).

Table (5): Distribution of Clostridium difficile infection in relation to types of the drugs.

Drugs		Positive PCR		Negative PCR		P value
		No.	%	No.	%	
Ceftriaxone	Yes	2	66.7	1	33.3	0.184^{NS}
	No	14	29.8	33	70.2	
Cefotaxime	Yes	9	24.3	28	75.7	0.04^{*}
	No	7	53.8	6	46.2	
Imuran(azathioprine)	Yes	3	75	1	25	0.055^{NS}
	No	13	28.3	33	71.7	
Azithromycin	Yes	1	33.3	2	66.7	0.959^{NS}
	No	15	31.9	32	68.1	
Ampiclox	Yes	7	50	7	50	0.089
	No	9	25	27	75	
Meropnem	Yes	2	100	0	0	0.035^{*}
	No	14	29.2	34	70.8	
Pentasa (mesalamine)	Yes	4	80	1	20	0.015^{*}
	No	12	26.7	33	73.3	
Gentamycin	Yes	1	33.3	2	66.7	0.959^{NS}
	No	15	31.9	32	68.1	
Amikacin	Yes	1	50	1	50	0.578^{NS}
	No	15	31.3	33	68.8	

Discussion

C. difficile is an organism that can be found in most of people's without causing symptoms, but in some people, it can cause a severe colitis. C. difficile toxin damages the fragile lining of the bowel causing loose watery bowel movements [15,16].

In current study, the included cases were presented with different clinical feature. These results are in accordance with those obtained by La Mont and Thraka [17] who showed that the clinical picture of C. difficile infection (CDI) may vary from asymptomatic carriers, with various severity of diarrhea to the most severe, life-threatening forms of colitis. Moreover, patients with IBD tend to have an unusual presentation of C. difficile associated diarrhea (CDAD). Our results showed the majority of positive cases15 (32.6%) have no history of hospitalization in the last 3 months.

That suggest most cases enrolled in current study were classified as nosocomial acquired infection, this result was similar to many other investigations working in this field as study done in Iraq by Angham [18] and with broad studies by (Sadeghifard et al. [19]; Szajewska et al. [20] who found that C. difficile was the most widely recognized agent saw in more than 52 % of episodes related with nosocomial diarrhea in hospitalized patients, with respect to some geographical variation, diarrhea is to be account for 1 to 14% of all nosocomial infections all through the world [21].

In our study 16 out of 50 patients hospitalized in AL-Kadhimiya teaching hospital were positive tcdB gene encoded toxin B and negative for tcdA gene encoded toxin A, results in current study revealed that C. difficile-associated disease where toxin A (-), toxin B (+). A study done by (Drudy D et al [22] showed that that toxin A is not essential for disease, while toxin B is essential for disease. The finding that toxin B and not toxin A is a basic for C. difficile disease ailment distinct difference a conspicuous difference to prior examinations performed utilizing purified toxin preparations, which had led to the hypothesis that toxin A was the major virulence factor of C. difficile [23]. However, a study in Iraq revealed that the toxins A/B have an important role in the development of the disease [24].

One of the interesting finding in many studies that toxin A-B+ strains cause the same spectrum of disease as toxin A+B+ isolates, ranging from asymptomatic carriage through to pseudomembranous colitis. There is a propensity towards more severe disease in patients infected with toxin A-B+ strains [23, 25]. Although it is not clear why increased severity is associated with these strains one of the possible explanation for such results is that the absence of anti- toxin A- immune response, which play important role in controlling disease severity [26].

Result obtained in this study show a statistical significant (P=0.034) association between C. difficile infection with

inflammatory bowel disease (IBD) The IBD is an independent risk factor for CDI, with an approximately 3-fold increased risk compared with non-IBD patients. The most Important factor in the development of C. difficile associated disease (CDAD) in patients with IBD is antibiotic therapy. Around 60% of patients with IBD report the utilization of anti-microbials before the advancement of CDAD [27].

The current study showed that the most commonly used antibiotic agent was Cefotaxime (24.3%). There was significant relation between positive results with Cefotaxime, Meropenem and Pentasa (Mesalamine).This result may be suggested that the use of β-lactam antibiotics facilitates the overgrowth of C. difficile in the gastrointestinal tract and the possible explanation for such results is that severe alterations of the gut microbiome with loss of resistance to colonization against C. difficile are thought to be the major trigger for CDAD and this quite accord with abroad studies of Knecht., et al [28].

Studies on different immunosuppressants and immunomodulators, for example, Pentasa and azathioprine, 6-mercaptopurine are vague or have just been done in little gatherings, yet some theory recommend that the impact of immunosuppressants and immunomodulators causes alteration on intestinal microbiota which improve development of C. difficile [29].

Conclusion

Clinical features of C. difficile infections (CDIs) are different and ranging from asymptomatic to various severity of diarrhea. There is an association between IBD and its exacerbations with (CDIs). Clinically the toxin B alone elicits severe enterotoxic effects increase rate of C. difficile infections (CDIs).

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