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ORIGINAL ARTICLE

Clostridium difficile Occurrence, Toxin Profile and Antibiotic Susceptibility: An Egyptian Center Experience

[1]Rehab H. El-Sokkary, [1]Marian A. [1]Gerges, Hend E. Sharaf, [1]Lobna Abdel Fattah, [1]Fatma A. Amer, [1]Rehab M. Elsaid Tash, [2]Rania Aamir1, [2]Ashraf M. Sherif*, [3]Mohamed S. Hegab, [3]Shaimaa S. A. Elashkar, [3]Atef M Khalil , [4]Ghada M. Al-Akad, [5]Tamer S. Salah

[1]Department of Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt; [2] Department of Pediatrics, Faculty of Medicine, Cairo University, Egypt; [3]Department of Pediatrics, Faculty of Medicine, Zagazig University, Egypt; [4]Department of Clinical pathology, Faculty of Medicine, Zagazig University, Egypt; [5] Anesthesia and ICU, Zagazig Faculty of Medicine, Zagazig University, Zagazig, Egypt

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Corresponding Author:
Ashraf M. Sherif
Assistant Professor of Pediatrics, Faculty of Medicine, Cairo University, Egypt
ashraf36@hotmail.com

ABSTRACT

Background: *Clostridium difficile (C. difficile) infection (CDI) is of concern in healthcare facilities. The role of binary toxin alone is controversial. Resistance to antibiotic therapy has emerged.* ***Objectives:*** *to determine the occurrence of C. difficile, their toxigenicity, antibiotic susceptibility pattern and the effect of binary toxin gene.* ***Methodology:*** *A cross section study, stools samples were collected from 211 patients. Strains were identified, antibiogram was determined using ATB™ ANA, and E-test for vancomycin. A multiplex PCR assay for (tpi), tcdA and tcdB genes, followed by a duplex PCR for cdtA and cdtB genes were done.* ***Results:*** *C. difficile was isolated from 29 samples (13.7%).. One strain was resistant to metronidazole, and three had intermediate susceptibility. All isolates were positive for (tpi) gene. Fifteen isolates (51.7%) were non toxigenic and 14 (48.2%) were toxigenic. Three toxigenic isolates harbored binary toxin genes. It was not possible to identify any impact of binary toxin due to small number found to harbor it.* ***Conclusions****: C. difficile is not a major problem in our locality. Further studies are needed to detect the role of binary toxin. Proper infection control measures and rationale use of antimicrobials are mandatory to prohibit further exaggeration of the problem.*

INTRODUCTION

Clostridium difficile (*C. difficile*) is one of the most important causes of health care-associated infections and the leading cause of intestinal infections related to antibiotic consumption. These infections range from diarrhea to life-threatening inflammation of the colon, sepsis, and ultimately death. Risk factors for CDI include duration of hospital stay, underlying illness, age, and previous use of virtually any antimicrobial drug, mostly cephalosporins and fluoroquinolones [1].

Epidemic and clinically important types of *C. difficile* are evolving that have been associated with increased virulence, resistance to antibiotics or both [1,2]. Moreover, the increasing morbidity, mortality and relapse rates, along with the emergence of community-associated disease have made this organism of significant concern [3]. The pathogenicity of *C. difficile* depends on the action of one/two large cytotoxins (A and B) acting as glycosyltransferases which disrupt the actin cytoskeleton by modifying guanose triphosphatases within the intestinal epithelial cells [4]. The two major *C.difficile* toxins are encoded by the genes *tcdA* and *tcdB* respectively. A third toxin, binary toxin (CDT), has been described in some *C. difficile* strains which is an ADP-ribosyltransferase that disrupts the structure of actin cytoskeleton in epithelial cells [5]. It also induces the formation of microtubule-based extensions on epithelial cell surface leading to increased adherence [6]. CDT is encoded by the genes *cdtA* and *cdtB*. While toxins A and B are recognized as the main virulence factors, the role of binary toxin alone in *C. difficile* pathogenesis is unclear though it may play an adjunctive role to toxins A and B [7].

Surveillance data from different Zagazig University Hospital departments were reported to the infection control unit. The detection of *C. difficile* was not performed as a routine test for hospitalized patients complaining from diahrrea. Thus this study was designed to determine the occurrance of *C. difficile* isolates, their toxigenicity, the antibiotic susceptibility pattern and the effect of binary toxin gene (if present) on susceptibility pattern and disease outcome.

METHODOLOGY

This cross sectional study was conducted in Zagazig University Hospitals, a large university hospital

in the eastern province of Egypt, from October 2015 to May 2016. It was approved by the ethical committee of Zagazig University, and a written informed consent from all participants or their guardians was provided in accordance with the Declaration of Helsinki.

Enrolled patients were selected by systemic random sampling from those admitted to inpatient units with the highest rates of diarrhea as revealed from patients' records. These clinical units were Pediatrics, General Surgery, Anesthesia and Intensive Care and Clinical Oncology. Patients were included if they were hospitalized patients suffering from health-care associated diarrhea. Diarrhea was defined as passing unformed stools for more than 3 times/24h [2]. The infection was considered as health-care associated if the patient symptoms began at least 72h after hospital admission [8]. Patients' personal and relevant clinical data were obtained including age, sex, severity of diarrhea, clinical course, antibiotic history, presence of other comorbidity and mortality, previous hospital admissions and antibiotics use.

Isolation and identification of *C. difficile*

Stool samples were collected from included patients [9]. Specimens were submitted to the Infection Control Laboratory in the same day. If any delay was anticipated, samples were kept at 4˚C. Stool samples were exposed to an alcohol shock procedure [9], cultured on *C. difficile* agar base (Oxoid Ltd, England) supplemented with *C. difficile* agar supplement (Oxoid Ltd, SR0096) giving a final concentration of cefoxitin (8 g/L) and D cycloserine (250 g/L) and incubated anaerobically at 37˚C for 48-72h. Suspected colonies were identified by morphology, odor and by being positive in latex agglutination test (Oxoid™ *Clostridium difficile* Test Kit). Isolates were kept in tryptic soy broth (Oxoid Ltd, England) with 20% glycerol at -20˚C until further use.

Antibiotic susceptibility testing

All *C. difficile* isolates were tested for susceptibility to 12 antimicrobials using ATB™ ANA (bioMérieux® SA, France) according to the manufacturer's instructions. In addition, the minimal inhibitory concentration (MIC) of vancomycin was determined using Etest (bioMérieux, Craponne, France) on *Brucella* blood agar. One to 1.5 McFarland suspension of the organism in tryptic soy broth was used in Etest according to the manufacturer's instructions. Results of Etest were interpreted according to Clinical and Laboratory Standard Institute (CLSI) guidelines [10].

DNA extraction

This was done using QIAamp DNA Stool MiniKit (Qiagen, Hilden, Germany) after three repeats of boiling and freezing (10 min each).

PCR for *tcd*A and *tcd*B genes

Each isolate was subjected to a multiplex PCR assay targeting triose phosphate isomerase (*tpi*) gene (a species-specific housekeeping gene), in addition to both *tcd*A and *tcd*B genes coding for toxin A and toxin B, respectively. Each reaction was performed using 5 µl template DNA, 25 pmole of each primer, except for *tpi* forward and reverse primers where 12.5 pmole were used, and 12.5 µl Taq PCR Master Mix solution (2X) in a total volume of 25 µl. Primers sequences and cycling conditions were done as previously mentioned [11].

PCR for binary toxin genes

All isolates were also subjected to a duplex PCR test targeting *cdt*A and *cdt*B genes coding for the enzymatic and the binding components of binary toxin, respectively. PCR reactions were performed in a total volume of 50 µl containing 1X Taq PCR Master Mix solution (25 µl), 20 pmole of each primer, 0.1% TritonX-100 and 5 µl of template DNA. Primer sequences and cycling conditions were done as previously mentioned [12]. All PCR reactions were performed using Biometra thermal cycler (Germany). *C. difficile* ATCC® BAA-1870 was used as a quality control reference strain.

Statistical analysis

The collected data were encoded and analyzed using SPSS version 16. The power of the study is 80% i.e., the probability of error is 20%, confidence interval is 95% and level of significance < 0.05. Quantitative variables were given as mean ± SD, and range for summarization and student t test. For qualitative data, number and percentage of observation at each category were used for summarization and chi-square for analysis.

RESULTS

General characters of the study population

Data collection phase took around 4 months. By using systemic random sampling, every 6th patient that fulfilled the criteria of inclusion was enrolled for the study. The sample size was 211; 43 (20%) from surgery unit, 41 (19.4%) from ICU, 25 (11.8%) from orthopedic unit, 65 (30.8%) from oncology unit and 37 (17.5%) from pediatric oncology unit (Table 1). All patients had abdominal pain. Fever was present in 93 (44.1%) patients. History of broad spectrum antibiotic uptake prior to the development of diarrhea was present in 198 (93.8%) cases. One hundred and two patients (48.3%) were treated with antineoplastic agents prior to the development of diarrhea.

Table 1. Frequency distribution of isolated strains

	Non* C. difficile** ***(n=182)	**C. difficile** ***(n=29)***	
Age range in years (mean ± SD)	3-90 (44.9 ± 2.4)	7-85 (52.2 ± 2.3)	t=1.51 P= 0.13
Sex No. (%)			
-Male	100 (54.9)	17 (58.6)	X^2= 0.14
- Female	82 (45.1)	12 (41.4)	P=0.71
Site of admission No. (%)			
- Surgery unit	35 (19.5)	8(27.6)	X^2= 2.25
- ICU	37 (20.3)	4 (13.8)	P=0.69
- Orthopedic	23 (12.6)	2(6.9)	
- Oncology	55 (30.2)	10 (34.5)	
- Pediatric oncology	32 (17.6)	5(17.2)	

The frequency of occurrence of *C. difficile* infection in the study population

A total of unrepeated 211 stool samples were collected from equal number of hospitalized patients. *C. difficile* was isolated from 29 stool samples giving a ratio of 13.7% among the studied patients (Table 2). All isolates were positive for the triose phosphate isomerase (*tpi*) gene.

The frequency of occurrence of toxin A and toxin B genes in *C. difficile* isolates

Of the 29 *C. difficile* isolates, 15 isolates (51.7%) were non toxigenic (harbored none of the examined toxin genes) and 14 (48.2%) had one or more of the examined toxin genes (toxigenic). The frequency of isolation of toxigenic strains did not differ significantly from that of non-toxigenic one regarding the age (P 0.27), sex (P 0.88), or the admission unit (P 0.35) of studied patients (Table 2).

Table 2. Frequency distribution of toxigenic and non toxigenic *C. difficile* strains

	Toxigenic *(n=14)*	*Non toxigenic* *(n=15)*	
Age range in years (mean ± SD)	7-85 (57.3 ± 2.0)	9-76 (47± 2.6)	t=1.14 P= 0.27
Sex No. (%)			
-Male	8 (57.1)	9 (60.0)	X^2= 0.02
- Female	6 (42.9)	6 (40.0)	P=0.88
Site of admission No. (%)			
- Surgery unit	3 (27.4)	5(33.3)	X^2= 8.9
- ICU	4 (28.6)	0	P=0.35
- Orthopedic	2 (14.3)	0	
- Oncology	4 (28.6)	6 (40.0)	
- Pediatric oncology	1 (7.1)	4(26.7)	

The frequency of occurrence of binary toxin genes in *C.difficile* isolates

Out of 29 *C.difficile* isolates, three isolates (10.3%) harbored binary toxin genes. All three isolates were obtained from female patients. The first one was 70-year female patient in ICU suffering from ventilator-associated pneumonia and she was treated with ceftriaxone 1 g/day + ampicillin/sulbactam 1.5 g/12hours + metronidazole prior to the development of diarrhea. The second one was 41-year female patient in the surgery unit that had perforated appendix operation and was on a course of ceftriaxone 1gm/day. The last one was 66-year female patient in the oncology unit that had cancer head of pancreas and was under weekly treatment by gemacitabine 1.8 g.

Antibiotic susceptibility pattern of isolated *C.difficile:*

All tested isolates, were susceptible to vancomycin with MIC ≤ 2µg/ml. High resistance ratios were recorded to cefotenan and imipenem (Table 3). Only one toxigenic, CDT-negative strain was resistant to metronidazole. Three toxigenic strains had intermediate susceptibility to metronidazole; only one was CDT-positive.

Along the study period, four deaths were recorded. All of them had toxigenic strains but negative for CDT.

Table 3. Susceptibility of CDT-positive and CDT-negative *C. difficile* isolates to antimicrobial agents used in ATB-ANA test

	cdt*A*/cdt*B* (+/+) (*n*=3)	cdt*A*/cdt*B* (-/-) (*n*=26)	*P*
	Resistance ratio No. (%)	Resistance ratio No. (%)	
Benzyl penicillin	0 (0)	3 (11.5)	0.72
Amoxicillin	0 (0)	3 (11.5)	0.72
Piperacillin	0 (0)	2 (7.7)	0.80
Co-amoxiclav	0 (0)	0 (0)	-
Ticarcillin-clavulinic acid	0 (0)	0 (0)	-
Piperacillin-tazobactam	0 (0)	0 (0)	-
Cefotenan	3 (100)	21 (80.8)	0.55
Imipenem	3 (100)	20 (76.9)	0.48
Chloramphenicol	0 (0)	2 (7.7)	0.80
Clindamycin	2 (66.7)	14 (53.8)	0.58
Metronidazole	0 (0)*	1 (3.8)**	0.89

*One strain (33.3%) had intermediate susceptibility.
**Two strains (7.7%) had intermediate susceptibility.

DISCUSSION

No much data is available regarding the role of *C. difficile* in causing diarrhea among hospitalized patients in the investigated hospitals. Moreover, there are few epidemiologic data published on *C. difficile* in Arabian countries over the last 15 years [13]. This is partly attributed to limited technologies and facilities for culturing and identifying *C. difficile* specifically and anaerobic pathogens, generally. In this study, the isolation frequency of *C. difficile* among studied patients was 13.7%. Although this comes consistent with previous reports from other countries inside and outside the Middle East (Jordan 13.7%, Kuwait 10.5%, Iran 21% and South Africa 14%) [14-17], it appears much higher than what has been recorded in other studies (6.1- 6.8% in Iranian studies and 4.9-9.5% in Saudi Arabia) [18-20]. In a previous Egyptian study[21], the isolation rate from diarrheic patients was 57.5%, an obviously higher figure than this study. The discrepancies in incidence reports may be partly due to differences in the regulations of antibiotic consumption. Anyhow, this result calls for more rationale use of antimicrobials in hospitals. Further studies are needed concerning the contribution of other enteric pathogens in causing diarrhea among hospitalized patients to find the exact role of *C. difficile* in that issue.

The relation between CDI and elderly patients has been confirmed previously [1]. The risk of infection was reported to be as much as 20-fold higher in patients ≥ 65 years old than for younger patients [22,23]. However, there are increasing reports of CDI in younger individuals, including people that would be considered at low risk of infection [3,24]. In this study, the mean age of affected patients was 52.2±2.3 years. Moreover, the highest ratios of infection, 24.1% and 18.4%, were observed in patients between 50 and 60 years of age and those above 70 years, respectively, which comes consistent with previous reports.

The pathogenicity of *C. difficile* is mediated mainly by the presence of two large clostridial toxins TcdA and/or TcdB. In our study, nearly half of *C. difficile* isolates (48.2%) were found to be toxigenic which is consistent with a previous study in South Africa [17], as well as, with a previous Egyptian study where 69.6% of total *C. difficile* isolates were toxigenic[21].

In addition to the large clostridial toxins, TcdA and TcdB, several strains isolated from outbreaks and severe infections have been shown to harbor the genes encoding CDT. The interest in CDT-producing strains of *C. difficile* has developed in the last few years. Since that time, CDT-producing strains have been isolated from patients throughout Europe and Asia indicating that such strains are widespread [5,12,25,26]. Three of our *C. difficile* isolates harbored CDT genes. Previous reports have shown wide discrepancy regarding the incidence of CDT genes among *C. difficile* isolates ranging from 1.6% in Japan and Korea[26] to 6.4% in UK hospitals [8], 8.6% in Poland [25] and 10.3% among toxigenic strains in France [12]. The impact of these genes and whether they undergo expression has not been assessed in our study, as until now, no CDT clinical assays are available [27].

As previously reported [12,25], CDT genes were detected only in toxigenic strains which goes in accord with our results. No obvious link was observed between the three strains harboring CDT, isolated in our study

To find out if the presence of toxins has any impact on the antimicrobial susceptibility pattern of *C. difficile* isolates, we carried out *in vitro* susceptibility tests using the ATB-ANA test, which is less time-consuming and less cumbersome than the standard agar dilution method. Furthermore, it produces results comparable to that of reference methods as reported previously [28]. Only one strain was resistant to metronidazole and it was found to be toxigenic but CDT-negative. Moreover, three strains had intermediate susceptibility to metronidazole; all of them were toxigenic and only one was CDT-positive. No resistance has been recorded to vancomycin in Etest.

Previous studies have shown that more severe CDI, regarding abdominal pain and diarrhea, was more frequent with CDT-producing *C. difficile* strains. Moreover, mortality and attributable mortality was 2.5 times higher with these strains [29]. we couldn't conclude any statically relation between those infected with CDT harboring genes and the disease severity

It worth mentioning that getting the stools samples was not easy. Perhaps this is the outcome of three reasons. First, clinicians are not too much concerned as regards the etiology of diarrhea among their patients, and emphasis is placed on empirical treatment, which is usually successful. That is particularly important in the background of the high efficiency of metronidazole (one of the anti-diarrheal agents used in our hospital); identified in our work. Second is the limited financial support available, which is more accentuated by the current economic situation in Egypt. Third is the fact that studying *C. difficile* at a molecular level is costly. In spite of this, the current work is providing an educational experience to all healthcare providers, and is throwing light and drawing attention to a neglected nosocomial pathogen. It can also sensitize other working groups in our facility to launch more resources and carry out larger studies.

In conclusion, *C. difficile* infection is prevalent among hospitalized patients in the investigated hospitals. So far, the magnitude of the problem is not so great. Further studies are needed to detect the role of binary toxin. Proper infection control measures and rationale use of antimicrobials are mandatory to prohibit further exaggeration of the problem. Being one of the first reports on the occurrence of binary toxin genes among *C. difficile* isolates from patients in the investigated hospitals, and due to the limited data about *C. difficile* and their toxin profile from our area, further studies should be planned involving higher number of patients.

Strengths of the study:

First: The variability of the included patients enrolled from different clinical units. Second: The toxogenicity of the isolates were detected by presence of corresponding coding genes rather than immunoassay. Third: It is a contribution to epidemiological data regarding the occurrence of *C. difficile* in the Arabian countries, as there are few epidemiologic data published on *C. difficile* in this part of the world over the last 15 years.

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