Full Length Research Paper

Prevalence of Clostridium difficile among cases of antibiotics associated diarrhea in hospitalized patients in an Egyptian hospital

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Background and aim of the work: Clostridium difficile associated diarrhea is an important nosocomial infection that occurs predominantly after hospitalization and administration of broad spectrum antibiotics. In this work we aimed to determine the prevalence of toxigenic C. difficile among cases of AAD in Cairo university hospitals in Egypt using specific Polymerase Chain Reaction protocols. Patients and methods: Stool samples were collected from 100 adult cases (19-59 years old) admitted in wards and ICUs of Cairo- University hospitals in Egypt, clinically suffering from AAD and twenty healthy individuals as a control group and subjected to direct microscopy examination, culture on blood agar media and chromognic culture media for C. difficile and finally molecular detection of C. difficile DNA using multiplex Polymerase chain Reaction of triose phosphate isomerase gene tpi, tcdA and tcdB genes which code for toxin A as an enterotoxin and toxin B as a cytotoxin respectively and cdtA and cdtB as an enzymatic and binding component respectively of binary toxin. Results: Two out of 100 cases were positive for the ctdB gene (toxin B) and tpi gene and one of the two positive cases was also positive for cdtA and cdtB (binary toxin). No cases were positive for tcdA toxin A. Regarding control group no samples were positive for any of the tested genes. Conclusion: This study confirms the accuracy and reliability of PCR methodology in the detection of toxigenic C. difficile, offering combined species identification and toxigenic type characterization, and suggests that C. difficile is responsible for a small, but underappreciated, proportion of antibiotic associated diarrheal cases in our country, and further study on a large scale is warranted in this area.

Keywords: C. difficile ; DNA isolation; Molecular diagnosis; Health care acquired infection

INTRODUCTION

Clostridium difficile is an obligate anaerobic, spore-producing, gram-positive rod that was first described in 1935 (Bartlett et al., 1977). Its link with pseudomembranous colitis and Clostridium difficile–associated diarrhea (CDAD) was established in 1978 (Poutanen and Simor 2004). It is the implicated pathogen in 20% to 30% of patients with antibiotic-associated diarrhea, 50% to 75% of those with antibiotic-associated colitis, and more than 90% of those with antibiotic-associated pseudomembranous colitis (Kelly et al., 1994). C. difficile remains the most commonly recognized cause of infectious diarrhea in healthcare setting and is increasingly important as a community pathogen (Bartlett 2006). CDAD is an important nosocomial infection that occurs predominantly after hospitalization and administration of broad spectrum antibiotics especially

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affecting the elderly and believed to be associated with an increase in length of hospital stay, cost and substantial morbidity and mortality (Wilcox et al., 1998). CDAD is usually diagnosed by detection of C. difficile cytotoxin in stool samples or by isolation and confirmed identification of toxigenic C. difficile in a stool culture (Ticehurst et al., 2006).

Although there are no regional or national Clostridium difficile infection (CDI) surveillance data for long term care facilities, patients in these settings are often elderly and have been exposed to antimicrobials, both are important risk factors for CDI, suggesting that rates of disease and/or colonization could potentially be high (Stamper et al., 2009). The C. difficile infection is not a reportable case in Egypt, so that there are few surveillance data. A few reliable techniques for toxin detection are available in Egyptian clinical laboratories. Polymerase chain Reaction (PCR) techniques appear to be reliable means of diagnosing CDI.These techniques could overcome the requirement for fresh specimens and provide a more sensitive test, specific for each *C. difficile* toxin (Perry et al., 2010).

In this study, we aiming at determining the prevalence of toxigenicC. difficile among the cases of AAD in hospitalized Egyptian population and the profile of toxigenicity of the strains presented in the stools, we developed, in this study, the PCR protocolsspecific to the tcdA (toxin A), tcdB (toxin B), cdtA and cdtB (binary toxin: CDT) genes to identify the C. difficlein patients suffering from bloody diarrhea.

PATIENTS AND METHODS

Ethical issues. The research protocol was approved by the Research Ethics Committee of the Cairo University hospital in Egypt. Informed oral consent was obtained from all participants.

The present study was conducted on 100 patients selected from inpatients of Kasr Al Aini hospital wards and ICUs. The patients clinically suffering from antibiotics associated diarrhea, loose or liquid stools were collected from patients suspected of having C. difficile related diarrhea due to the presence of suggestive criteria: antibiotic usage for 5days-1week, mild to moderate bloody diarrhea, cramping pain in lower part of abdomen, presence of vomiting or fever . Twenty healthy individuals who came for routine checkup, without suffering from any diarrhea or intestinal problems, and did not receive antibiotics, were chosen as a control group. A checklist was filled for each patient and control case.

All patients (cases and control) were subjected to: Full history taking, including the following; frequency of diarrhea, +/-Abdominal pain, +/- Abdominal tenderness after antibiotic intake or presence of diarrhea, leukocytosis, +/- fever, +/- vomiting.

Sample collection. Two to three mililiters of stool was taken from each patient or control person in a vacuum packed vacutainer to keep anaerobic conditions. Each stool sample was subjected to routine laboratory investigations in the form of direct microscopic examination for detection of leukocytes, Red blood cells (RBCs) and pathogenic microorganism. Also, conventional culture on Blood, MacConkey and Salmonella-Shigella agar. Chromogenic culture media for C. difficilewere used to confirm the identification of toxigenic strains (ChromAgar-France) (C. difficult base,C. difficult supplement) (Version 1, 101207). It was prepared according to the manufacturer's instructions. All media were incubated in an anaerobic workstation at 37°C for a full 24-h period and then removed into the air so that colony counts could be performed. After a maximum of 30 min in air, culture plates were re-incubated anaerobically for a further 24hours.All colonies, irrespective of appearance were initially investigated by Gram stain, and suspect colonies were further confirmed by their characteristic morphology and natural yellow-green colony fluorescence under long-wave UV light. Generation of blue fluorescence under long-wave UV light at 365 nm, indicates a positive reaction on chromogenic media (Perry et al., 2010).

*Genomic DNA purification.*Genomic DNA was extracted directly from the stool samples using the DNA Extraction kit from a Favorgen Biotech corporation company, Taiwan *(Cat.No.FASTI 001).*

PCR assays. Specific multiplex PCR protocols were used as described by (Lemee et al., 2004), targeting a species-specific internal fragment of the triose phosphate isomerase (tpi) housekeeping gene, toxin A gene (tcd*)* and toxin B gene (tcdB). The presence of the binary toxin was ascertained by two different reactions using two different primer pairs for the enzymatic and the binding components of the cdt gene A and B respectively. Amplification was performed for each toxin using primer sequences as shown in Table 1.

A control gene was used in all runs as an internal control for Clostridium difficile (triose phosphate gene), its positivity was indicated by the appearance of a band at 230 bp (Samie et al., 2008; Costa et al., 2012).

Detection of the PCR products. The PCR products were observed under UV illumination after electrophoresis in 2% agarose gel and stained with 0.5 µg/mL of ethidium bromide [15].Molecular weight marker. (100 bp DNA Ladder) is provided by Fermentas (Catalog No.: #SM0241, LITHUANIA), concentration 0.5 µg/µL, supplied with 1 mL 6× DNA Loading Dye, stored at -20 ºC.The sizes of distinctive amplicons differed sufficiently to be distinguished from one another using agarose gel electrophoresis (Table 1).

Table 1. Primer sequences and gene targets for molecular characterization of C. difficile.

Targeted gene	Gene function	Primer	Sequence	Product size (bp)	Ref.
Triose phosphate isomerase (tpi)	C. difficile specific housekeeping gene	Tpi-F tpi-R	AAAGAAGCTACTAAGGGTACAAA CATAATATTGGGTCTATTCCTAC	230	[10]
tcdA	Toxin A, enterotoxiceffectson epithelial cells	tcdA (NK2) tcdA (NK3)	CCCAATAGAAGATTCAATATTAAGCTT GGAAGAAAAGAACTTCTGGCTCACTC AGGT	251	[11]
tcdB	Toxin B, cytotoxic effects on epithelial cells	YT17 YT18	GGTGGAGCTGCTTCATTGGAGAG GTGTAACCTACTTTCATAACACCA	399	[12]
cdtA of binary toxin	Enzymatic component of binary toxin	cdtAFor cdtARev	TGAACCTGGAAAAGGTGATG AGGATTATTTACTGGACCATTTG	376	[9]
cdtB of binary toxin	Binding component of binary toxin	3cdtBFor 3cdtBRe v	GATTCACAGCTAATGTAACTA TATAGTCTGACCAACTATTTC	475	[9]

The statistical Analysis. Data were statistically described in terms of the mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann Whitney U test for independent samples. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. P values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

Demographic information on the study population.

Table 2 shows all data of patient and control group.

Clinical diagnosis of selected patients revealed acute myeloid leukemia in 17% (17/100) of cases which is the highest incidence, followed by liver cirrhosis in 8 /100 cases (8%), HSM in 7 /100 cases (7%) peptic ulcer in 6 /100 cases (6. Clinical diagnosis of the two positive cases were Pemphigus vulgaris and liver cirrhosis with P- value =0.612.

The distribution of patients within inpatient wards, were as follows 47/84cases (56%) were admitted in internal medicine wards, followed by cesium that included 21/84 cases (25%), then surgical wards with 13 /84 cases (15%) and finally chest wards with 3/84 cases (3%).

The distribution of patients in ICUs revealed that the majority was in internal medicine ICUs 10 cases/16 (62.5%), while surgical ICUs had 6 cases/16 (37.5%). The two positive cases were admitted in medical ICUs.

Timing of diarrhea in relation to hospital admission date was as follows: 15 /100 cases (15%) started diarrhea 5 days after hospital admission; 11 /100 cases (11%) started 4 days after admission entry; 8/100 cases (8%) started 6 days after hospital admission entry; 7 /100 cases (7%) diarrhea started after 3 days admission. It was observed that the majority of study cases started diarrhea after 5 days of hospital admission. The two positive cases as provided by PCR started diarrhea 3 and 6 days after hospital admission respectively, with P-value 0.323.

Soft stool showed the highest rate (50%), while liquid stool specimens showed the lowest rate (15%) among our studied cases. The two positive samples were soft stool samples with P-value >0.050.

It was found that 38 /100 cases (38%) showed 4 motions /day; 28 /100 cases (28%) showed 3 motions/day; 14 /100 cases (14%) showed 5 motions /day and 12 /100 cases (12%) showed 6 motions /day; while all cases of the control

Table2. Individual demographic, clinical and laboratory data of patients and control group.

| Item		Demographic data	
		Control group	Patient group
Gender	Male	40%	52%
	Female	60%	48%
Age range (year)	14	0%	1%
	15-59	90%	74%
	≥60	10%	25%
	Average	32	47
Clinical data (risk factors)			
Fever		No	47%
Colics with diarrhea		No	73%
Bloody diarrhea		No	45%
Laboratory data			
Direct examination			
High leukocytic count/HPF in stool		No	100% leucocytic
High RBCs /HPF in stool		No	77% RBCs
PCR			
Toxin B		Negative	2% Positive
Binary toxin		Negative	1% Positive
Triose phosphate gene		Negative	2% Positive
Chromogenic culture			
		No growth	No growth
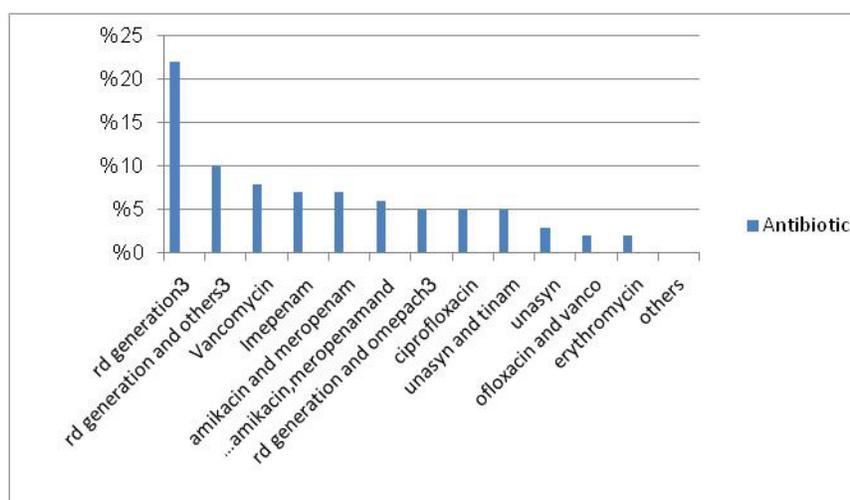
Figure 1. The most commonly received antibiotics among patient group.

group had normal bowel habits. Our two positive cases showed 3 and 4 motions/days respectively.

Diarrhea was accompanied by pain and colic in 72/100 cases (72%), while in 28 /100 of cases (28%) the diarrhea was painless; fifty percent of positive cases were accompanied by colic and pain with an insignificant P value between positive and negative cases P-value 0.469.

Forty five /100 cases (45%) of patients had bloody diarrhea, while in 55 /100 of cases (55%) of them the diarrhea was bloodless. Fifty percent of positive cases suffered from bloody diarrhea with an insignificant P value between positive and negative cases (P- value = 1.000).

Figure 1 shows that the most commonly received antibiotics among the patient group was the 3rd generation
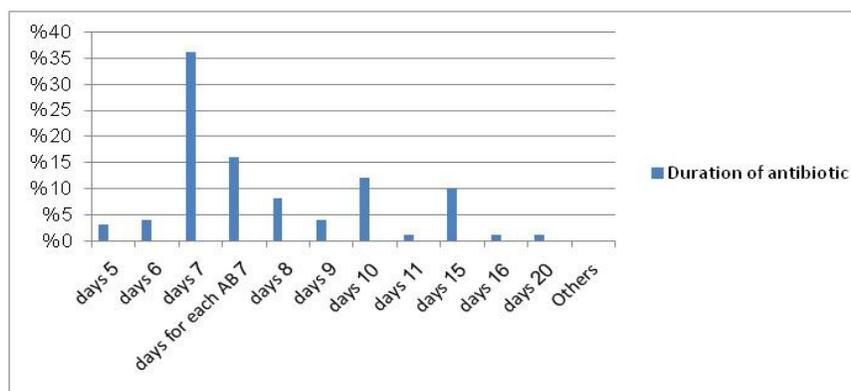
Figure 2. Duration of antibiotic intake in patient group**.**

Table 3. Isolated microorganisms from stool culture on conventional media

Culture	Number of cases	Percentage
Pure Staphylococcus aureus	15	15
Pure Candida spp.	10	10
Pure Proteus spp.	1	1
Pure Salmonella spp.	1	1
Normal flora	73	73
Total	100	100

Table 4. Results of multiplex PCR for 100 stool samples of patient group

Toxin	Band size (bp)	Cases	
		Number	Percentage
Toxin A	251	Zero	0
Toxin B	399	2 cases	2
Binary toxin	376, 475	1 case	1
Triosephosphate gene	230	2 cases	2

cephalosporins either alone or in combinations with other drugs in 37/100 cases (37%) followed by vancomycin in 8/100 cases (8%), and Imepenam in 7/100 cases (7%). One of the two positive cases was receiving the 3rd generation cephalosporins and the other one was receiving maxipime with insignificant P values in all antibiotics between positive and negative cases.

According to the duration of antibiotics intake figure 2 showed that the commonest antibiotic duration among selected cases was about 7 days in 36 /100 cases (36%). The two positive cases were receiving antibiotics for 9 days and 7 days respectively.

Regarding the timing of diarrhea in relation to antibiotic intake, 19/100 cases (19%) started diarrhea after 4 days of antibiotic intake, while 16 /100 cases (16%) after 3 days of antibiotic intake, 11 /100 cases (11%) after 2 days; 7 /100 cases (7%) after 10 days of antibiotic intake. The two positive cases started diarrhea after 3 and 6 days respectively from antibiotic intake with P value: 0.612.

Molecular study of Clostridium difficile toxins using multiplex PCR.

Multiplex PCR was used for detection of Clostridium difficile toxins in stool samples including toxin A (enterotoxin), toxin B (cytotoxin) and Binary toxin. Interpretation of PCR results done for stool samples showed that no stool samples of cases or control groups were positive for toxin A; while 2/100 cases (2%) were positive for toxin B and only 1/100 case (1%) was positive for Binary toxin; and Triose phosphate gene was positive in 2/100 cases (2%) as shown in Figures 3 and 4. No stool samples in the control group were positive for any of the studied toxins by PCR. The results were summarized in Table 4.
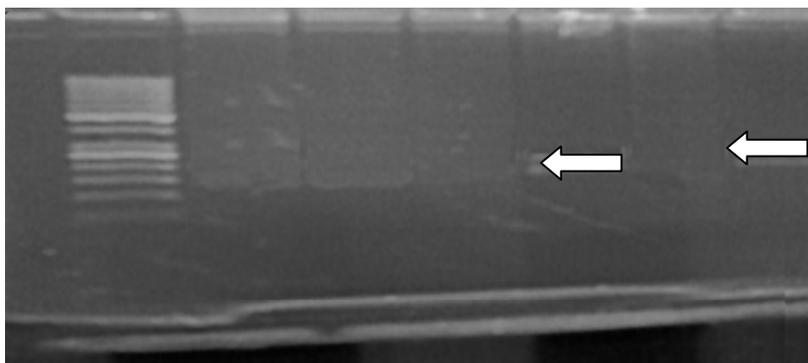
Figure 3. **Positive cases for toxin B and triose phosphate gene by PCR**
Lane no. 1; Showing molecular weight marker (100-bp ladder)
Lane no. 5; Showing band at 399 nm indicating toxin B and
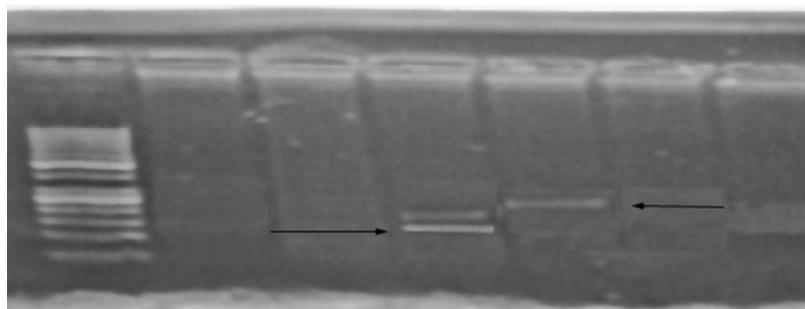Lane no. 7; Showing band at 230 nm for triose phosphate gene.

Figure 4. **Positive cases for binary toxin and triose phosphate gene by PCR**
Lane no. 1; Showing molecular weight marker (100-bp ladder),
 Lane no 4; Showing band at 376 nm for enzymatic component of binary toxin, and band
at 230 nm for triose phosphate gene .
Lane no.5; With band at 475 nm for a binding component of binary toxin

DISCUSSION

Clostridium difficile is the main etiological agent of antibiotic associated diarrhea and pseudo membranous colitis, and is also the major recognized cause of nosocomial diarrhea (Polage et al., 2012; Bartlett 1990). There are increasing reports of community-associated CDI, including disease in younger individuals and people with few or no traditional risk factors (Kuntz et al., 2011). One of the major risk factors for *C. difficile* infection is advanced age, so it was an important selection criteria in our study. The 100 selected patients included 75% adults and 25% elderly, which was different from the study of (Samie et al., 2008) who selected his cases 80% adults and 20% children ranging from 1 month to 15 years. Many studies have proven that elderly patients were found to have a higher prevalence CDI, since these patients are more susceptible due to co-morbidities, co-existing chronic infections, inflammatory or neoplastic disease (Boone et al., 2012). Positive cases in our study were adults and this

was in agreement with the previous study (Samie et al., 2008) where most of positive cases were adults.

In our study, the patient group suffered from diarrhea, cramping pain and sometimes fever 39°C-40°C and that was in agreement with the selected patients in previous study [20], who also suffered from colitis as well as watery diarrhea, lower abdominal pain and cramping, with or without low grade fever, and leukocytosis.

The antibiotics most frequently implicated in diarrhea associated with *C. difficile* infection are clindamycin, expanded-spectrum penicillins, and cephalosporins. However, virtually any antibiotic may be implicated, including brief courses of antibiotics that are given prophylactically before surgery (with the exception of parenteral vancomycin). Occasional cases follow treatment with methotrexate or paclitaxel for cancer chemotherapy (Bélanger et al., 2003). A great proportion of the selected cases in our study (37%) received the 3rd generation cephalosporin either alone or in combinations with other types of antibiotics,While the two positive cases proofed by

PCR received the 3rd and the 4th generation cephalosporines respectively.

In our study, the symptoms of the two positive cases during the setting of antibiotic intake revealed one case that started diarrhea after 4 days of antibiotic intake, while the other started after 6 days and this was in agreement with a previous study (Cohen et al., 2010), who found that in most cases the symptoms occurred in the setting of antibiotic administration. The symptoms may begin during antibiotic therapy or 5 to 10 days after the antibiotic administration, and also stated that in few cases symptoms may present as late as 10 weeks after cessation of therapy.

In the present study the prevalence rate of infection was 2%. Also, in a previous study (Samie et al., 2008), a low prevalence rate (7.1%) was mentioned as all cases in that study were not laboratory identified and were chosen randomly based on clinical criteria. While only the positive cases evidenced by immunoassay were chosen for further work up in another study (Boone et al., 2012), so the prevalence rate was the highest (100%).

The stool samples of the chosen patient group showed variety inconsistency, some were soft (50%), some were semi formed (20%), others were liquid (30%) and none of them were formed. The positive two stool samples were soft and that was in agreement with a previous study (Boone et al., 2012), where the chosen stool samples, including liquid, partially formed and soft specimens and his positive samples were soft or liquid respectively. Other authors (Goldstein et al., 2009), found that the liquid and formed specimens had similar rates, a finding that was surprising, since most of the formed specimens were from patients who did not have diarrhea and were asymptomatic. Some authors (Cohen et al., 2010), recommended testing liquid or partially formed, soft specimens and formed specimens.

Our two positive cases received acid-reducing medication which was similar with the cases reported in previous studies that an association of CDAD in patients receiving stomach acid-reducing medication (i.e., proton pump inhibitors and antacids) has been reported by (Dial et al., 2004; Yearsley et al., 2006). Additional studies are needed in order to learn more about the protective benefit of stomach acid against this disease and particularly against spores.

Findings that are considered nonspecific, but suggestive, of *C. difficile* infection includes fecal leukocytes (Guerrant et al., 2001). This was in agreement with our results where the two positive samples proofed by PCR subjected to direct examination revealed high leukocytes and RBCs content.

The two PCR positive cases in our study showed an increased number of daily motions up to 3 and 4 times per day respectively and this was in agreement with a previous study (Miller 2007), that stool samples suggestive of CDAD had blood or pus in the stool with increasing number of motions that may reach up to 10-15 times /day.

In the present study, 100 random stool samples were cultured directly on conventional media in the form of blood agar, MacConkey, S.S. agar, and chromogenic culture media for detection of positive cases of *Clostridium difficile*, while other studies subjected their stool samples to alcohol shock before inoculation onto blood agar (Martirosian 2006).

As *C. difficile* readily forms spores, various treatments such as heat shock and alcohol shock may be applied to specimens for culture to reduce or eradicate vegetative cells and hence limit the growth of contaminating flora. Alcohol shock in particular has been shown to increase the isolation frequency of *C. difficile* from stool samples in some studies (Marler et al., 1992).

Perry *et al.,*[8] cultured only previously checked specimens by immunoassay for the presence of *Clostridium difficile* and the positive samples cultured in chromogenic agar gave a high prevalence rate of 100% and this is in contrast to our study, where none of the positive cases by PCR gave fluorescence on chromogenic culture media using a 365nm UV lamp even after repetition of the culture for 3 times.

In spite of the advantages of ELISA in being fast (2-6 hour), easy to perform, with a high specificity, but it has also well-recognized limitations in that the sensitivity and specificity are slightly lower and occasional specimens may contain inhibitory substances or low prevalence of the organism and sometimes degradation of toxins occur in the sample that give false negative results (Samie et al., 2007).

An explanation for negative growth on chromogenic agar medium is that there were no more viable cells of C. difficile in the sample, but the PCR would still be a positive detecting presence of genes.

The most important advantage of PCR in the clinical microbiology field is the rapidity that it offers for pathogen diagnosis. To date, some PCR assays have been developed for the specific detection of C. difficile in feces samples. Multiplex PCR was performed in the present study showing, 2% of samples were triose phosphate isomerase (tpi) positive which is an internal gene in Clostridium difficile bacteria and its presence indicated that the sample contain C. difficile, 2/100 cases (2%) were positive for toxin B and 1/100 case (1%) was positive for binary toxin and that was in contrast with the study done by (Samie et al., 2008), which showed a higher incidence where 13% were positive for tpi , 7% of cases were positive for at least one toxin , 5.5% of cases were toxin A positive ,6.5% of cases were toxin B positive and 3.7% of cases were positive for binary toxin.

Data presented above has proved that it is difficult to diagnose C. difficile associated diarrhea because of its low prevalence in Egypt and that was proved also by a large study done by NAMRU 3 in cooperation with faculty of

medicine Cairo University (internal medicine and Monira hospital), Faculty of medicine of Alexandria and faculty of medicine of Jordon from may 2010 to may 2011, which revealed that no cases were detected in Kasr Al Aini, only one case in Alexandria and only one case in Gordon, using ELISA for detection of toxins.

Some explanations for the low prevalence of CDAD in Egypt were mentioned, but it needs further investigations to be proved. The first explanation is that although antimicrobial drugs leading to disruption of the normal intestinal microflora leads to C. difficile overgrowth (Pepin et al., 2005), yet this does not occur in Egypt as C. difficileis not prevalent as normal flora in the gastrointestinal tract may be due to difference in dietary habits between Middle East population, Europeans and Americans.

The second explanation is because of the usage of chlorine as an antiseptic in Egypt which kills spores of C. difficile, but in Europe and United States they don't use chlorine, the two explanations were mentioned by a study done by NAMRU3.Other standard precautions for infection control including hand hygiene and aseptic techniques are carefully applied and continually observed in the Egyptian hospitals according to the national Egyptian guideline of infection control may also play a role in its low prevalence in Egypt. (Prevention of Hospital Acquired Infections (PHAI) 2002).

The third explanation is that several studies have recovered spores of C. difficile from food products including retail meat (Rodriguez-Palacios et al., 2007; Costa et al., 2012). Contamination of carcasses during slaughter and processing is most likely (Jobstl et al., 2010) and the presence of dormant spores of the bacterium in the muscle tissues of food animals should also be considered. Also concerns about zoonotic and food borne transmission of CDI has been heightened by the apparent increase in CDI in humans caused by C. difficile (Bauer et al., 2011).

CONCLUSION

We can conclude that this study confirms the usefulness of PCR methodologies in the detection of toxigenic C. difficile and suggests that C. difficile is responsible for a small, but underappreciated proportion of diarrheal cases, and further studies are warranted in this area, but taking in consideration the budgetary issues as well as the challenge of extracting nucleic acids from feces and separating template DNA from potentially interfering substances.

There was no specific data in the two positive cases detected by PCR in comparison to other negative cases and the P-value was not significant between positive and negative cases regarding all collected data except intake of omepac where there is a significant association between positive and negative cases P value is 0.040. So CDI must

be suspected in any hospitalized case suffering from diarrhea after antibiotic intake and direct examination of stool sample revealed high leukocytes and RBCs or occult blood especially in the elderly, in association of a comorbidity and receiving acid reducing drugs.

Limitation of the study

The study must have been done on a larger scale and with the usage of immunoassay as a screening method before PCR with more connections and cooperation between wards.

Also using the Qiagenkit for extracting DNA can be the cause of low yielding, especially for anaerobic bacteria.

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