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Diagnosis of *Clostridium difficile* infection in patients with hospital-acquired diarrhea

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Clostridium difficile infection (CDI) is a rapidly emerging infection that may have devastating consequences. Prompt and accurate diagnosis is crucial for management and control. The aim of this study was to determine the incidence of *C. difficile* associated diarrhea among hospitalized patients, and to compare different diagnostic laboratory methods for detection of toxin producing strains in clinical specimens. The study was conducted at a university hospital in Cairo during the period from May 2013 till June 2015. Subjects were under antibiotic therapy and presented with hospital-acquired diarrhea. Four hundred and sixty-five stool specimens were processed by different microbiological methods. *C. difficile* was recovered in culture in 51 of stool specimens. Of these, 86.3% to 98% were positive for toxin production by 2 different methods. This study showed that antibiotic intake is the major risk factor for development of hospital-acquired diarrhea. We evaluated different microbiological methods for diagnosis of *C. difficile*. We recommend the use of toxigenic culture as a gold standard for microbiological diagnosis of *C. difficile*.

Keywords: *Clostridium difficile*, hospital-acquired diarrhea

Clostridium difficile, a Gram-positive, spore-forming anaerobe, is the leading cause of healthcare-associated, antibiotic-associated diarrhea. Infection occurs among patients whose intestinal microbiota have been disrupted by prolonged treatment with broad-spectrum antibiotics, allowing *Clostridium difficile* to colonize the compromised gastrointestinal tract (Dembek *et al.*, 2015). *C. difficile* infection (CDI) is associated with a wide range of clinical manifestations, from asymptomatic colonization to mild diarrhea or more severe pseudomembranous colitis that may progress to toxic megacolon, intestinal perforation, sepsis and death (Vincent and Manges, 2015).

Since *C. difficile* can be isolated from stool in asymptomatic patients, culture alone is not adequate to diagnose CDI and may misdiagnose antibiotic-associated diarrhea caused by other agents, unless stool samples are also assayed for the presence of *C. difficile* toxins. The recent emergence of epidemic, hypervirulent strains has reinforced the need for cultivation of *C. difficile* for subsequent typing, molecular studies, and determination of antimicrobial susceptibility. A variety of media exist for recovery of *C. difficile* from fecal samples or rectal swabs, but there are very few studies that compare media and culture conditions (Stevens *et al*., 2015).

The aim of this study was to determine the incidence of *C. difficile* associated diarrhea among hospitalized patients, and to compare different diagnostic laboratory methods for detection of toxin producing strains in clinical specimens.

Materials and Methods

The study was conducted on children (1 month~18 years of age) with hospital-acquired diarrhea at the Children's hospital, Ain Shams University Hospitals. Patients who developed diarrhea

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during the period from May 2013 and June 2015 were included in this study. Hospital-acquired infection was confirmed if the patient developed diarrhea after more than 3 days of symptom-free hospital stay.

Four hundred and sixty-five episodes of diarrhea were recorded during this period. The patients were subjected to thorough history taking and clinical examination.

Specimens

Stool specimens were examined for consistency, color and presence of mucus. Data regarding risk factors (comorbidity, previous gastrointestinal surgery, exposure to antibiotics; types, and duration, proton pump inhibitors, presence of nasogastric tube and receipt of chemotherapy) was also recorded.

Cases not receiving antimicrobial treatment, or those who had received antimicrobial medication within two weeks prior to admission, and those who were not admitted to hospital (as children observed in out-patient clinics and emergency department) were excluded.

Microbiological evaluation

Non-duplicated specimens (at least 10 ml of feces) were collected from patients. Samples were treated with 96% ethanol and inoculated onto cefoxitin-cycloserine-fructose agar (CCFA) plates (Oxoid). These were incubated for 5 days in an anaerobic Gas-Pack system (Oxoid). Colonies were identified phenotypically and chemotaxonomically (Summanen *et al*., 1993; Johnson *et al*., 2007). *Clostridium difficile* toxin was detected in stool by a Xpect® *C. diffcile* toxin A/B enzyme immunoassay (Tenover *et al*., 2011) and tissue culture cytotoxicity assay (Solomon *et al*., 2013).

CDI was defined as the presence of diarrhea (three or more stools in 24 h), a stool test result positive for toxigenic *C. difficile* and a positive stool toxin assay either Xpect® *C. difficile* toxins A and B (Remel) or Cytotoxicity assay.

Statistical methods

Univariate *P* values were calculated from Chi-square tables and from Mann Whitney test using SPSS version 20 (IBM). Multiple logistic regression models were fitted for risk factor analysis of *C. difficile* infection and for predicting outcomes. $P < 0.05$ was considered as statistically significant.

Results

Of 465 children with diarrhea enrolled in the study, 60.9% were male; their median age was 30 months (IQR 14-72). Fifty-one children (11%) met the definition for CDI. *C. difficile* toxins A and B were positive in 44 (9.5%) and cytotoxin assay was positive in 50 (10.7%) of cases.

Bacteriological culture

Among 465 stool specimens, 21.9% showed no growth and 78.1% yielded positive culture (363 specimens). Of these, 404 isolates were recovered. Identification of isolates revealed 317 (78.5%) Gram-positive anaerobes, 64 (15.8%) Gram-negative anaerobes, and 23 (5.7%) *Candida* spp. Fig. 1 shows the distribution of micro-organisms isolated in the study.

Detection of *C. difficile* and its toxins

C. difficile isolates were identified microscopically by Gram stain and macroscopically by characteristic colonial morphology, horse odor and hemolysis on Columbia blood agar (Collee *et al*., 1989). *C. difficile* isolates were identified biochemically by lipase, lecithinase, catalase, indole production; gelatin and esculin hydrolysis; and glucose, fructose, lactose, maltose, sucrose and mannitol fermentation (Collee and Marr, 1996).

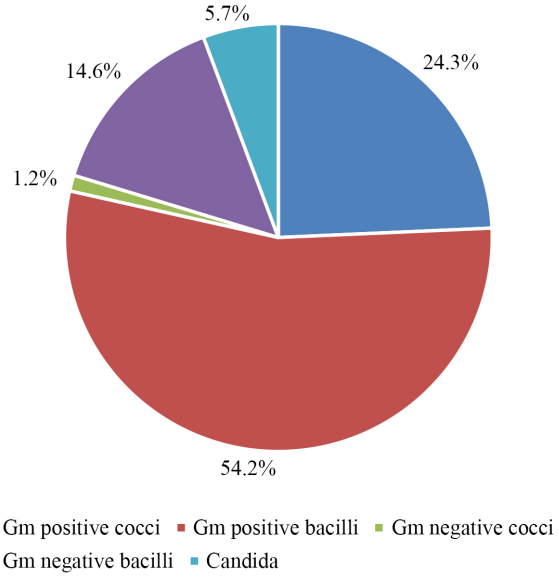

Fig. 1. Distribution of micro-organisms isolated in the study.

Toxigenic culture (TC)

Of 465 stool specimens, 51(11%) were positive by TC. The organism was typically identified based on Gram stain; flat, filamentous, colony morphology (pale yellow color), "horse barn" odor and characteristic yellow-green fluorescence under UV light (Fig. 2).

Only specimens that showed positive results by TC were tested for *in vivo* toxin production by 2 methods (EIA and CTA).

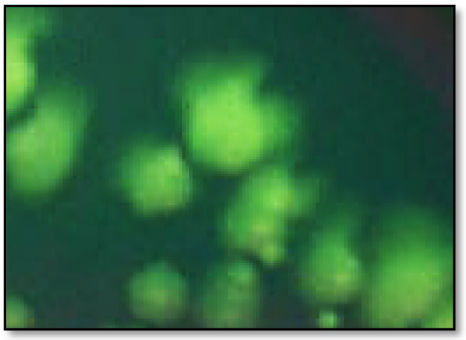

Fig. 2. *C. difficile* colonies under UV light.

Enzyme immunoassay (EIA)

Of the 51 *C. difficile* isolates, 44 were positive for *C. difficile* toxin A/B production by EIA (Fig. 3 and Table 1). In nine patients with CDI, EIA was unable to detect the toxin directly from the stool specimen; instead, it was detected from brain

Fig. 3. Detection of toxigenic isolates of *C. difficile* by EIA. (A) Positive EIA showing 2 colored lines of any intensity, one in the test region and the other in the control region. (B) Negative EIA showing only one colored line in the control region.

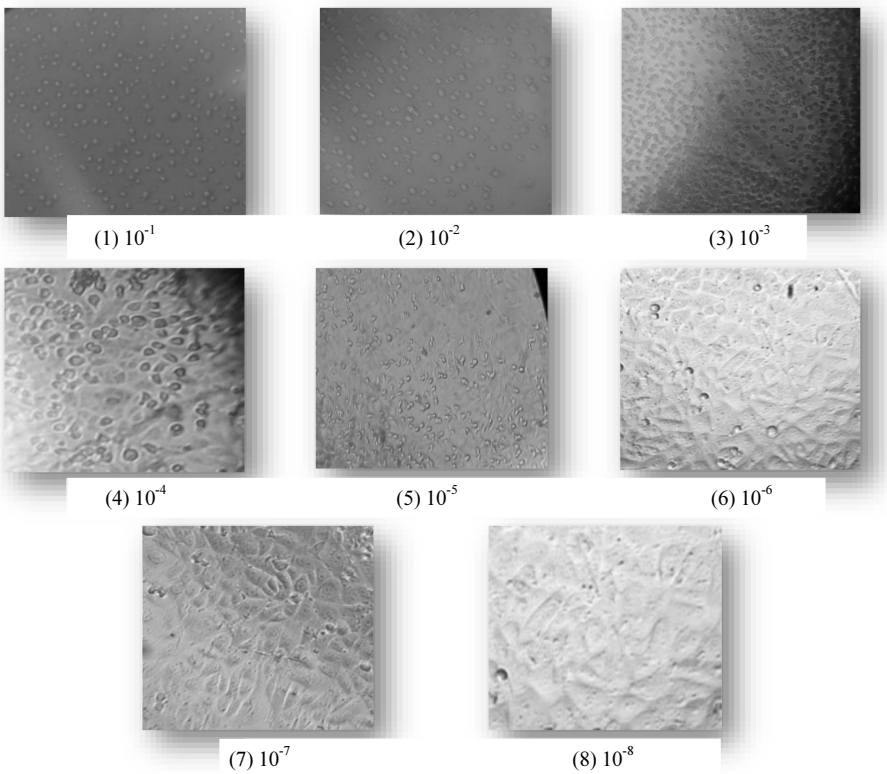

Fig. 4. Cytotoxicity induced by *C. difficile* toxins. (1) Cytopathic effect at dilution 10^{-1}, (2) - (8) Cytopathic effect at dilution 10^{-2}–10^{-8} respectively.

heart infusion broth culture.

Cytotoxicity assay (CTA)

In the present study, among 51 isolates of *C. difficile*, 50 (98%) were positive for toxin production by CTA and only one (2%) was found to be non-toxin producing.

Risk factors

Antibiotics exposure : Table 2 shows that antimicrobial classes with broad-spectrum activity, including 4th generation cephalosporins (P = 0.001), aminoglycosides (P < 0.001), levofloxacin (P = 0.032), meropenems (P = 0.012), and vancomycin (P = 0.002), were associated with CDI.

Table 3 shows that the duration of antibiotic administrated during hospitalization was significantly longer in *C. difficile* positive cases than negative cases.

Antibiotic combination was significantly more frequent in *C. difficile* positive cases than negative cases (Table 4). Almost

Table 1. Frequency of toxin producing *C. difficile* by EIA

Test	Positive		Negative		Total	
	No.	%	No.	%	No.	%
EIA	44	86.3	7	13.7	51	100

Table 3. Duration of antibiotics administration among patients with and without CDI

Antibiotics duration	Infection		Z	P
	C. difficile	Other		
Median (days)	18	10	7.294	< 0.001*
IQR (days)	15 - 25	8 - 15		

IQR: inter-quartile range

Table 2. Correlation between antibiotic exposure and CDI

Antibiotic type	Infection				X^2	P
	C. difficile		Other			
	No.	%	No.	%		
Other antibiotics	47	10.7	394	89.3	-	0.320
Macrolides (n=24)	4	16.7	20	83.3		
Other antibiotics	50	10.8	414	89.2	-	0.110
Tigecycline (n=1)	1	100	0	0		
Other antibiotics	24	10.6	203	89.4	0.071	0.790
Amoxicillin (n=238)	27	11.3	211	88.7		
Other antibiotics	39	9.4	375	90.6	9.257	0.002*
Vancomycin (n=51)	12	23.5	39	76.5		
Other antibiotics	44	10.7	367	89.3	0.249	0.618
Imipenem (n=54)	7	13.0	47	87.0		
Other antibiotics	47	10.4	406	89.6	6.310	0.012*
Meropenem (n=12)	4	33.3	8	66.7		
Other antibiotics	48	10.5	410	89.5	-	0.032*
Levofloxacin (n=7)	3	42.9	4	57.1		
Other antibiotics	49	11.0	398	89.0	-	1.000
Piperacillin (n=18)	2	11.1	16	88.9		
Other antibiotics	48	10.9	394	89.1	-	0.730
Ciprofloxacin (n=23)	3	13.0	20	87.0		
Other antibiotics	27	7.5	332	92.5	19.161	< 0.001*
Aminoglycosides (n=106)	24	22.6	82	77.4		
Other antibiotics	27	8.0	311	92.0	11.251	0.001*
4th generation Cephalosporin (n=127)	24	18.9	103	81.1		
Other antibiotics	21	12.4%	149	87.6%	0.527	0.468
3th generation Cephalosporin (n=295)	30	10.2%	265	89.8%		

Table 4. **Antibiotic combination among patients with and without CDI**

Number of antibiotics	Infection				X^2	P
	C. difficile		Other			
	No.	%	No.	%		
1	2	1.9	103	98.1		
2	20	8.0	230	92.0		
3	20	21.7	72	78.3	59.319	< 0.001*
4	6	40.0	9	60.0		
5	3	100	0	0		

Table 5. **Association between CDI and different risk factors**

Risk factor		Infection				X^2	P
		C. difficile		Other			
		No.	%	No.	%		
Chemotherapy	Absence	46	90.2	389	94.9	-	0.358
	Presence	5	9.8	25	6.0		
Proton Pump Inhibitors	Absence	18	35.3	186	44.9	1.711	0.191
	Presence	33	64.7	228	55.1		
Previous gastrointestinal surgery	Absence	47	92.2	388	93.7	-	0.558
	Presence	4	7.8	26	6.3		
Nasogastric tube	Absence	42	82.4	361	87.2	0.922	0.337
	Presence	9	17.6	53	12.8		

98% of the patients with CDI received intensive antibiotic therapy with two to five antibiotics.

Other risk factors : Table 5 shows no significant association between CDI and different risk factors included in the study.

Discussion

Human *C. difficile*-associated diarrhea is responsible for one-fourth of all antibiotic-associated diarrhea cases, with approximately three million cases per year (Rineh *et al*., 2014).

Antibiotic exposure is the primary risk factors for CDI because of disruption of gastrointestinal microbiota diversity (Argamany *et al*., 2015), which may increase vulnerability to colonization of the gastrointestinal tract with *C. difficile*, with a proportion of colonized individuals progressing to CDI (Mylonakis *et al*., 2001). All patients included in this study were receiving antibiotic therapy during hospitalization.

CDI is caused by toxigenic *C. difficile* that usually produces 2 major toxins, toxins A (enterotoxin; TcdA) and B (cytotoxin; TcdB) (Kim *et al*., 2014). Since 2000, this infection has become increasingly prevalent, and more severe forms of the disease have emerged. Large hospital outbreaks have required ward closures and extensive infection control measures (Bomers *et al*., 2015).

Thus, rapid diagnosis of CDI is a key step in the successful management of the disease. It enables the physician to initiate treatment of the patient and to implement control measures promptly to avoid cross-contamination. An accurate diagnosis is also essential to obtain reliable data for surveillance, to assess the efficacy of intervention measures to reduce CDI, and to enable comparison between institutions as part of performance management (Barbut *et al*., 2014).

The diagnosis of CDI remains a challenge for many clinical microbiology laboratories. Different tests have emerged to diagnose CDI, but no single test approach has been proven effective to satisfy the need of all patients, hospitals and clinical microbiology laboratories. The selection of *C. difficle* testing and the testing procedure are dependent on each laboratory, institution and patient population (Hansen *et al*., 2010).

Several laboratory tests are available for detection of *C.*

difficile or its toxins in the feces, including: cell cytotoxicity assay, toxin/antigen detection and detection of toxin genes by nucleic acid amplification tests (Jamal *et al*., 2014).

In this study, three different methods were used for detection of *C. difficile* or its toxins. These include toxigenic culture (TC), cell cytotoxicity assay (CTA) and Enzyme immunoassays (EIA). All stool specimens (465) were processed by TC, only specimens that showed positive results were subjected to other methods (CTA and EIA).

In the present study, 51 (11%) patients were positive for toxigenic strains by TC assay (39 specimens were positive by direct plating on selective media after alcohol shock and 12 specimens that were culture-negative were analyzed by using an enrichment broth for plating on selective agar). This agrees with the observation that broth enrichment and duplicate culturing may enhance the *C. difficile* culture-positive rate by 30% (Peterson *et al*., 2011).

It should be noted that there is no standard method for TC assays available, making it difficult to compare results from other studies. A wide variety of media and differences in isolation protocols, such as the use of alcohol shock and variations in incubation time, are common (Humphries, 2012).

Both toxigenic bacterial culture and toxin gene PCR detect toxigenic bacteria, while toxin assays detect *in vivo* toxin production; in the present study both EIA and CTA were used for testing *in vivo* toxin production.

EIA remains the most commonly used test for the diagnosis of CDI in humans and animals worldwide due to its ease of use, low cost (in comparison to CTA or PCR) and the fast turnaround for results (Peterson *et al*., 2011). Out of 51 *C. difficile* detected in the present study by TC, 44 (86.3%) were positive for toxin A/B by EIA (Table 1). All tests were repeated on every sample that yielded discordant results using enrichment broth, leading to a marginally better rate of concordance between the 2 tests. The previous finding is consistent with Shilling (2013), in which 16 of the 19 *C. difficile* isolates (84.2%) were toxin positive by EIA.

In the present study, Vero cells were used; this cell line is the most sensitive to A/B toxins, and a similar CTA protocol was used successfully in previous studies (Silva *et al*., 2013). Out of 51 *C. difficile* detected in the present study by TC, 50 (98%) were positive for toxin production by CTA.

For many years, the detection of A/B toxins in feces via CTA was considered the gold standard method for the diagnosis of CDI. Studies have then shown that TC is more sensitive; therefore, it has been used as the new gold standard method. However, both techniques are time consuming, laborious, and require trained personnel. Thus, commercial enzyme immunoassays EIA are currently the most widely used techniques for the diagnosis of CDI (Silva *et al*., 2014).

In the present study, the use of TC was proposed as a gold standard for detection of toxigenic *C. difficile*, as it provides high sensitivity and specificity.

When evaluating EIA results versus TC for detecting toxigenic *C. difficile*, the present study revealed that the sensitivity of EIA was 86.3%. This result is in accordance with a previous study by Barkin *et al*. (2012), who reported that the sensitivity of EIA was 86.1%. Planche *et al*. (2008) found EIA had a sensitivity of less than 75%, even with toxigenic culture as the reference standard. On the other hand, the study by Silva *et al*. (2014) showed that the three EIAs tested had low sensitivities, ranging from 61 to 68%. EIAs are unable to detect some newer CDI strains, including epidemic clones, further explaining the waning performance of EIAs (Peterson *et al*., 2011).

When evaluating CTA results versus TC for detecting toxigenic *C. difficile*, the present study revealed that the sensitivity of CTA was 98%. This result is consistent with results by Brown *et al*. (2011) and Ota and McGowan *et al*. (2012), in which the sensitivity of CTA was 100% and 96%, respectively. Other studies by Lalande *et al*. (2011), Barbut *et al*. (2014), and Silva *et al*. (2014) showed that the sensitivity of CTA (in comparison with TC) was 69.4, 75, and 73.9%, respectively.

In the present study, cytotoxin testing was performed using cell culture plates freshly prepared, and samples were tested starting at a low dilution (i.e., 1:10). Thus, cytotoxicity results were likely more sensitive than those obtained using higher starting dilutions, commercially prepared cell culture, or samples shipped a distance, during which toxins may be degraded.

When EIA results were evaluated versus CTA, the present study showed that 86.3% (44/51) of isolates were positive by both tests, while 11.8% (6/51) of isolates were positive by CTA and negative by EIA. The current study revealed that the sensitivity of EIA compared to CTA was 88% and there was a

significant difference between the two tests. Our results are consistent with Planche *et al*. (2013) in which CTA showed higher detection rate than EIA and the sensitivity of EIA compared to CTA was 66.9% and 83.2% (by using 2 different toxin EIA kits). Another study by Silva *et al*. (2014) showed that the sensitivity of EIA compared to CTA was 64%. The EIA requires 100−1000 picograms of toxin as compared to the ability of the CTA to detect less than 10 picograms of toxin, thus explaining the lower sensitivity of EIA.

Conclusion

This study showed that antibiotic therapy remains the main risk factor for developing CDI in children. Toxin EIA test is not a suitable test for diagnosis of CDI by itself because of its low sensitivity. CTA is much better correlated to clinical outcome as compared to the TC method. We conclude that the use of TC as a gold standard for detection of toxigenic *C. difficile* may be the most feasible solution, due to its high sensitivity and specificity.

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