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Rapid Assays for Detection of *Clostridium difficile* and Its Toxins in Hospitalized Patients

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In this study, the rapid detection of *Clostridium difficile* and its toxins (A/B) in admitting patients in hospitals were offered the possibility of prevention of the bacterium spread into other patients. This bacterium is the most common cause of hospital-acquired diarrhea in patients treated with antibiotics, chemotherapeutic agents and other drugs that alter the normal equilibrium of the intestinal flora. The Stool samples were cultured on specific agar media with anaerobically growth equipment. The *C. difficile* toxins A and B were detected in fecal samples by using enzyme-linked immunosorbent assay (EIA). In this study, there was a higher incidence of *C. Difficile* infection among the hospitalized patients in different hospital wards, especially in the renal ward, hepatic ward and oncology ward. The using of EIA technique for pathogen detection is very useful and significant method for the rapid detection of the *C. difficile* strains.

Keywords: *Clostridium difficile*, toxins A/B, renal patients, hepatic patients and oncology patients.

The *Clostridium difficile* is a gram-positive, spore-forming bacterium, usually spread by the fecal-oral route. It is non-invasive and produces toxins A/B that is causing disease. This disease is ranging either from asymptomatic carriage to mild diarrhea, colitis or pseudo-membranous colitis[1], but *C. difficile* emerged as a major enteric pathogen with worldwide distribution[2].

In the United States, *C. difficile* was nosocomial pathogen. In 2011 were identified 453,000 cases with *C. difficile* infection, in addition there were 29,000 death cases associated with *C. difficile* infection [3]. The Nosocomial risks of C. *difficile* infection was more than the cost of treatments and hospitalizations (4). Increasing the annual expenditures were approximately $1.5 billion in the United States[5].

The incidence of *C. difficile* infections among hospitalized patients were varied widely from different times and in different locations, but has generally been increasing to almost 15 cases per 1000 hospital discharges[6] and approximately 20 cases per 100,000 persons/year in the community[7].

The enzyme immunoassay (EIA) is more common diagnostic test today. It can be used to detect glutamated-hydrogenase (so-called common antigen) and/or major Toxins A/B. It is inexpensive, rapid and easy to perform. A drawback of EIA toxin tests is a lack of sensitivity, but conversely EIA tests, have better specificity, as they cannot distinguish toxigenic from nontoxigenic *C.*

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difficile. Glutamate dehydrogenase is a good screening test, but the positive specimens must be subjected to another test, that used to detect the toxin A and/or B or the toxin genes[8].

MATERIALS AND METHODS

Patients: This study carried out at Menoufia University hospital admitted patients in the period between December 2016 and March 2017. The 81 cases include 41 males (50.61%) and 40 females (49.4%). All patients were divided into three groups; group 1 was 25 patients from renal ward, group 2 included 33 patients from hepatic ward and the other cases in group 3 included 23 patients from oncology ward. All patients under the this study were classified into another two groups, diabetic group (N=46 cases) and non-diabetic group (N=35 cases). The inclusion criteria of adult men or women were 16-85 years old of patients with equal duration of admission to hospital wards (more than two days). The collected stool samples were diarrhea (N = 59), enema (N = 2) and semi formed stool samples (N = 19).

The age, sex, duration of hospital admission and drug usage (antibiotics and chemotherapy) is obtained from the patient's clinical data. The case history of diabetes, liver diseases, kidney impairment and cancer are confirmed by the histopathology data. Every patient fulfilling the inclusion criteria was allocated to the intervention group. The written consent was obtained from all patients prior to enrollment in the study and ethical committee of Faculty of Medicine Hospital Menoufia University approved the protocol, which was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Stool samples: The stool samples available are collected after the patient's admission in sterilized plastic containers and transported to the microbiology lab for the screening of the presence of *C. difficile* bacterium and A/B toxins.

METHODS

Culture technique: Stool samples collected one by one and cultured anaerobically on selective media agar base (M836) with culture supplement (FD010).

Gram stain: All slides of bacteria examined under the oil immersion lens.

EIA detection of toxin A/B: The RIDASCREEN® *Clostridium difficile* toxin A/B is an enzyme immunoassay for determining toxin A and toxin B, specifically and simultaneously in the stool samples of patients using monoclonal antibodies. The reliable results were taken after only 2 hours and the effect of therapeutic measures were taken promptly.

Statistical analysis: The data were collected, tabulated and analyzed by SPSS (statistical package for social science) version 17.0 on IBM compatible computer (SPSS Inc., Chicago, IL, USA). The highly significant P value if it was less than 0.001, significant if it was less than 0.05 and not significant if it was more than 0.05.

RESULTS

The stool specimens were collected from selected hospitalized 81 patients under this study, were classified into three groups by gender of males in renal cases males were 4% and females were 96.0% of hepatic cases males were 69.7% and females were 30.3%, in oncology cases males were 73.9% and females were 26.1%. The patient's duration before stool sample collection in hospital possible ranged from 5 to 10 days as follows; about 10 days to patients of the renal group, 5 days for the hepatic group and 8 days for oncology patients (table 1).

The consistency of stool samples in renal cases is 36.0% of semi-formed and 64% with diarrhea. In hepatic group patients stool consistency is 30.3% with semi-formed, 63.6% with diarrhea and 6.1% with an enema, but oncology patients the stool consistency are diarrhea 100% (table 2).

In this study among 81 studied cases 1 case its stool sample showed no growth of Clostridium difficile Agar media (1.2%), 80 cases their stool sample showed growth of gram-positive bacilli (98.8%). The bacterial growth was 100% in hepatic and oncology group, but was 96% in renal group patients (table 3). The bacterial toxin A/B production are highly elevated in hepatic and oncology groups, 69 cases showed the positive result for EIA for toxin A/B (85.2%) and 12 cases showed negative results for the same test (14.8%).

The positive bacilli grow in hepatic and oncology groups are showing highly significant growth in comparison of different groups with each other, but the toxin production is 56% positive and 44% negative toxin production in renal patients under this study (table 3).

The *C. difficile* growth is in diabetic patients in the different group was 97.8 % and 100% in non-diabetic patients. The possibilities of toxin A/B production are highly elevated in comparing with diabetic patients (table 4).

Table 5 appears gram-positive growth with highly significant increasing (94.1%) in chronic kidney disease without dialysis, but renal failure with dialysis group the growth are 100%. The toxin A/B production positivity is about 50% renal failure in dialysis cases and 58.8% of chronic kidney disease patients. The last table number 6 showed the different antibiotic and chemotherapy using in all patients of different three groups under this study with impaired pathogen growth.

DISCUSSION

The older age was independently associated with the development of severe complicated *C. difficile* infection in the hospitalized patients[9]. The most important primary risk factors include age more than 65 years, age less than 1 year with co-morbidity or underlying conditions[10]. The age over 70 years is an independent risk factor of severe *C. difficile* associated disease and adverse outcome, including death[11], age were positively associated with the incidence of *C. difficile* infection and its relationship to disease severity remains controversial[12]. This previous suggestion was agreed with our observation where was significantly a difference between hepatic and oncology cases as regards age, on the other hand, there was no significant difference between renal and oncology group (P value < 0.05). It was argued that older patients may not be able to mount an effective immune response to the *C.*

Table 1. Different patient's duration of the stay in hospital for all groups.

Duration	Renal group (1) (N = 25)	Hepatic group (2) (N = 33)	Oncology group (3) (N = 23)	Test of significance	P value
Days in hospital (M ± SD)	10.48±6.61	5.67±2.83	8.13±4.05	U 2.08** 0.12* 0.11*	 $< 0.05^1$ $> 0.05^1$ $> 0.05^3$

U (Mann Whitney U test), * (t- test), ** (Mann Whitney U test), M ± SD (mean ± standard deviation) & N (number of patients).[1]= comparison between renal cases and oncology cases.[2]= comparison between hepatic cases and oncology cases.[3]= comparison between renal cases and hepatic cases.

Table 2. Stool consistency in different patient groups.

Stool consistency	Renal group (1) (N = 25)		Hepatic group (2) (N = 33)		Oncology group (3) (N = 23)		Test of significance	P value
	N	%	N	%	N	%	X^2	
Semi formed	9	36.0	10	30.3	0	0.0	10.19#	$<0.05^1$
Diarrhea	16	64.0	21	63.6	23	100	10.64	$<0.05^2$
Enema	0	0.0	2	6.1	0	0.0	10.05	$<0.05^3$

= Fisher's Exact test.X^2 (Chi Square test), % (percentage) & N (number of patients).[1] = comparison between renal cases and oncology cases.[2] = comparison between hepatic cases and oncology cases.[3] = comparison between renal cases and hepatic cases

difficile infection [13] thus, leading to severe disease and poor outcome in the elderly. The male gender was associated with severe disease[14], as in our foundation the hepatic and oncology groups, male gender were 69.7% and 73.9 % respectively, in contrast, the renal group that has 96% females.

The highest incidence rate of *C. difficile* infection and acquired within the long-term care facility, were indicating a substantial degree of transmission[15], these infections can lead to major complications for the patient's health (16), these agreements with this study duration and hospital stay for patients in hepatic unit was 5.67 and for oncology ward is 8.13 and for renal ward was 10.48 days (table 1). The clinical importance of *C. difficile* toxigenicity founded in liquid stool samples of hospitalized patients and the possibility of asymptomatic carrying in 2% of patients with formed stool[17], the quantitative colony counts were sufficiently high to detect the bacterium irrespective of stool consistency and the semi-formed stool should be sought for the pathogen in symptomatic patients with frequent stools[18]. These are discussed, the majority of our collected stool samples were diarrhea; the minority of specimens are semi-formed and few with an enema (table 2).

The main of diagnosis is the detection of *C. difficile* toxins in a diarrheal sample, but a few laboratories made cultures of the organism. This combination of tests should include culture (with toxin testing of the isolate), demonstration of toxin directly from the feces and the detection of *C.*

Table 3. Growth on *Clostridium difficile* agar medium and toxin A/B production

Growth on agar media	Renal group (1) (N = 25)		Hepatic group (2) (N = 33)		Oncology group (3) (N = 23)		Test of significance	P value
	N	%	N	%	N	%	X^2	
No growth	1	4.0	0	0.0	0	0.0	0.94#	> 0.05[1]
Gram positive bacilli	24	96.0	33	100	23	100	1.34	> 0.05[1]
							4.05	> 0.05[3]
Toxin A/B production	N	%	N	%	N	%	X2	P value
Positive	14	56.0	32	97.0	23	100	13.13	< 0.001[1]
Negative	11	44.0	1	3.0	0	0.0	0.71#	> 0.05[2]
							14.6	< 0.001[3]

= Fisher's Exact test.
X2 (Chi Square test), % (percentage) & N (number of patients).
[1]= comparison between renal cases and oncology cases.
[2] = comparison between hepatic cases and oncology cases.
[3] = comparison between renal cases and hepatic cases.

Table 4. Growth on *Clostridium difficile* agar medium and toxin A/B in diabetic and non-diabetic groups

Growth on agar media	Diabetic (N = 46)		Non diabetic (N = 35)		Fisher's Exacts	P value
	N	%	N	%		
No growth	1	2.2	0	0.0	0.77	> 0.05[1]
Gram positive bacilli	45	97.8	35	100		
Toxin A/B production	N	%	N	%		
Positive	36	78.3	33	94.3	4.05*	< 0.05[1]
Negative	10	21.7	2	5.7		

FE = Fisher's Exact test. X2 (Chi Square test), % (percentage) & N (number of patients).
[1]= comparison between diabetic cases and non diabetic cases

difficile antigen[19] and this was in agreement with this study as we first used the culture technique then the demonstration of toxin using EIA technique. The EIA has a sensitivity ranging from 60 to 70% and specificity of 98%, but symptomatic patients with negative tests should be tested by another more sensitive method[20]. The stool culture is the most sensitive test and is essential for epidemiological studies[1].

The patients with diarrhea were detected positive for *C. difficile* toxins A/B in stool specimens by EIA screen test in cases 74 % with diarrhea, 23.5% with semi-formed stool and 2.5% from enema[21], these were supported our observed significant difference data of toxin A/B were 56.0% with renal, 97.0% with hepatic and 100% with oncology patients (table 3). Acute renal dysfunction

Table 5. Growth on Agar medium and toxin A/B production in chronic kidney disease patients with no dialysis and renal failure patients on dialysis

Growth on agar media	Chronic kidney disease (no dialysis) (N = 17)		Renal failure on dialysis (N = 8)		Fisher's Exacts	P value
	N	%	N	%		
No growth	1	5.9	0	0	0.49	
Gram positive bacilli	16	94.1	8	100		> 0.05[1]
Toxin A/B production	N	%	N	%	FE	
Positive	10	58.8	4	50	0.17	
Negative	7	41.2	4	50		> 0.05[1]

FE = Fisher's Exact test.

Table 6. Drugs used in patients of study groups with bacteria growth.

Drugs and Growth	Renal group (1) (N = 25)		Hepatic group (2) (N = 33)		Oncology group (3) (N = 23)		Test of significance	P value
	N	%	N	%	N	%	x^2	
No drugs	1	4.0	2	6.1	0	0.0	48.0	<0.001[1]
Antibiotics	24	96.0	31	93.9	0	0.0	56.0	<0.001[2]
Chemotherapy	0	0.0	0	0.0	15	65.2	0.12*	0.720[3]
Both	0	0.0	0	0.0	8	34.8		
Antibiotics	N=24	%	N=31	%	N=8	%	x^2	
Single	13	54.2	16	51.6	6	75.0	1.08*	>0.05[1]
Combined	11	45.8	15	48.4	2	25.0	1.41*	>0.05[2]
							0.04*	>0.05[3]
A type of antibiotic used	N=24	%	N=31	%	N=8	%	x^2	
Cephotax	12	50.0	16	51.6	4	50.0	4.0	>0.05[1]
Unasyn	1	4.2	0	0	2	25.0	8.81	>0.05[2]
Cephotax + unasyn	5	20.8	8	25.8	1	12.5	2.24	>0.05[3]
Cephotax +cipro	1	4.2	1	3.2	0	0.0		
Unasyn +cipro	2	8.3	1	3.2	0	0.0		
Other combination	3	12.5	5	16.1	1	12.5		

* = Fisher's Exact test.X^2 (Chi Square test), % (percentage) & N (number of patients).[1] = comparison between renal cases and oncology cases.[2] = comparison between hepatic cases and oncology cases.[3]= comparison between renal cases and hepatic cases

can be used to define severe *C. difficile* infection[22] and the dialysis patients have impaired host defense mechanisms and frequently require antibiotics for various infective complications[23], this appear the high growth bacterium in our renal patients (96.0%) in addition to low toxin A/B production (56.0%).

In our results were100 0% growth of *C. difficile* Agar medium and 97.0% were positive for the production of toxin A/B using the EIA test in agreement with previous studies[24]. In addition to the formerly suggestions, the *C. difficile* infection is common amongst liver transplantation (22%) patients, cirrhotic liver disease[25, 26] and cirrhosis-related complications[26, 27], although diarrhea on hospital admission has been reported for 13% cirrhotic patients with *C. difficile* infection[26]. Recent evidence suggests that liver disease patients have increased morbidity, mortality and health care costs[28].

The cancer patients have a higher risk for *C. difficile* infection as compared to non-cancer patients[29], this confirms the 100% growth and toxin production of oncology group of our work. In the other recent foundation, there were no cancer-specific factors were identified to be related to *C. difficile* carriage. However, a younger age and a longer hospital stay may represent the characteristics of more aggressive and immunosuppressive oncologic disease [30].

In this study we found that 8 cases from 25 cases were admitted to renal unit were renal failure in dialysis and 2 cases from these 8 cases were showed positive growth on Clostridium difficile and we found that, 50% of renal failure on dialysis cases including two cases of the previous showed positive test for toxin A\B and 50% of cases show negative test for toxin A\B and this was in agreement with a study which indicated that, the dialysis process might be at high risk for the development of *C. difficile* associated disease, especially if the symptoms develop in dialysis patients[31], this risk of pathogen infection increased hospital-associated morbidity and mortality was greater in dialysis than of chronic kidney disease patients not undergoing dialysis[32].

Our significant difference between diabetic and non-diabetic cases as regards the production of toxin A/B (P-value <0.05) and this was in agreement with a study which demonstrated that, diabetes is an important risk factor for recurrence of *C. difficile* associated disease[33]. In addition, the diabetes-related hospitalization increases the risk of recurrent bacterium infection[34].

The patients who take antibiotics are most at risk for developing *C. difficile* infections because the beneficial bacteria that are normally present in the human gut and protect against infection can be suppressed for several weeks to months, during this time the patients can get sick from *C. difficile* picked up from contaminated surfaces or spread person to person[35]. The *C. difficile* infection in cancer patients receiving chemotherapy is 2.3 to 8.2% of these patients develop severe intestinal colitis[36].

Risk factors for bacterium infection in immune suppressed cancer patients appear to be their frequent hospitalizations and receipt of chemotherapeutic agents and antimicrobials that make them more susceptible to this disease[37] and this was in agreement with our study as all oncology patients were received chemotherapy and show positive production of *C. difficile* toxin A/B. Some chemotherapeutic drugs such as; methotrexate and 5-fluorouracil are most commonly reported to be associated with pathogen infection risk may be from the drug's ability to cause intense intestinal mucositis [37]. Several other chemotherapeutic agents, including cyclophosphamide, doxorubicin, cisplatin, paclitaxel, and vinorelbine, have been associated with *C. difficile* infection[38]. This was confirmed the obtaining significant difference between renal and oncology cases as regards taken drugs (P-value <0.001), there was a highly significant difference between hepatic and oncology cases as regards taken drugs (P-value is <0.001). The taken drugs include antibiotics, chemotherapy or both.

This study must be introduced into the infection control units of many hospital in Egypt, specially in Faculty of Medicine Hospital, Menoufia University. It is concluded that, *C. difficile* bacterium is widely spread among hospitalized patients in hospital wards, renal ward (56%), hepatic ward (97%) and oncology ward (100%). The immunosorbent assay for the detection of toxin A/B is a significant technique for the rapid detection of pathogenic *C. difficile* strain in patient's stool specimen.

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