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ORIGINAL ARTICLE

Diagnostic Value of Simple Immune-chromatographic Test for Rapid Detection of Clostridium difficile Infection

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Key words:
CDI - X/pect C.difficile toxin A/B test – CCFA - C. difficile test Kit - Real time PCR

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ABSTRACT

Background: *Clostridium difficile is a very important cause of antibiotic-associated diarrhea and pseudomembranous colitis. Diagnosis of C. difficile mainly relies on toxin detection in stool specimens from individuals with suspected disease.* ***Objective:*** *is to introduce to our microbiology laboratory of a simple test that may be rapid, cheap and easily manipulated than conventional methods for effective diagnosis of C. difficile infection.* ***Methodology****: Stool samples from sixty eight hospitalized patients developing CDI like symptoms were subjected to culture on CCFA, detection of toxins A and/or B by X/pect test (directly from stool samples and from culture isolates) and Real time PCR for detection of tcdA/ tcdB toxin genes.* ***Results****: Toxigenic C. difficile was detected in (22.1%) of suspected cases using tcdA/ tcdB real time PCR which was the gold standard method in our study. The positive rate for the direct X/pect test was 13.2% and for the indirect test was 14.7%. The sensitivity of direct X/pect test was 60%, specificity was 100%, PPV was 100%, and NPV was 89.8% with 91.2% agreement between the direct assay and real time PCR. While, the validity values for the indirect test was 66.7%, 100%, 100% & 91.4% for sensitivity, specificity, PP and NP values respectively, with 92.6% agreement between both assays. Antibiotic intake and recent hospitalization were the most commonly encountered risk factors, followed by number of hospitalization days. Penicillins and cephalosporins were the most frequently associated antibiotics, followed by clindamycin* ***Conclusions:*** *Using X/pect test can combine accurate results with simple procedure that offers results within 20 minutes. Although it is accompanied with low sensitivity and high rate of false-negative results, X/pect test may be of great benefit to practitioners particularly when you need STAT testing or 24 hour/ 7 days coverage. Further, it can be used as a preliminary screening approach allowing patients to be treated early and correctly in order to shorten the duration of symptoms and avoid complications.*

INTRODUCTION

Clostridium difficile is an important cause of antibiotic-associated diarrhea; it causes significant illness and is a main cause of hospital-acquired infection. Two major toxins produced by *C. difficile*, toxin A and toxin B, the level of toxins produced relates to the severity of the disease [1]. Toxin A is an enterotoxin that causes tissue damage while toxin B is a more potent cytotoxin and has a direct cytopathic effect by depolymerizing actin filaments causing destruction of the cytoskeleton, this leads to damage for the colonic mucosa [2].

Infection mainly occurs in hospitalized patients and individuals with *C.difficile*-associated disease (CDAD). shed spores in their stool, which can survive in the environment for up to five months. Clinical features of CDAD are not simply distinguished from other gastrointestinal diseases, including chronic inflammatory bowel disease and ulcerative colitis. Infection with toxigenic *C. difficile* is potentially life-threatening; thus, rapid diagnosis is crucial, to allow clinicians to initiate appropriate therapy and implement preventive measures to control nosocomial spread [3].

Diagnosis is made primarily by detecting toxins in the stools of persons with suspected disease. Direct stool toxin assays include the cytotoxin assay (CTA) and enzyme immunoassays. Cytotoxin assay (CTAs) is the gold standard for diagnosis of *C. difficile* infection (CDI). The CTA includes exposing cultured cells to fecal extracts in the absence and presence of anti-toxin. Positive samples have a cytopathic effect on cultured cells that have not been treated with anti-toxin. However the poor sensitivity and the technical complexity of CYT, as well as the need for 24 to 48 h incubation has resulted in the widespread replacement of CYT with toxin immunoassays that provide results within minutes [4].

C. difficile can also be detected by culturing the organism under anaerobic conditions. This method has

high sensitivity, but is time consuming and can require more than three days. In addition; this approach does not distinguish between toxigenic and non-toxigenic strains. Further testing of isolates using enzyme immunoassays is needed to identify if the strain is toxin-producing [5].

Glutamate dehydrogenase (GDH) antigen of *C. difficile* has been used as an alternative approach and GDH EIAs have been described to be highly sensitive for detection of *C. difficile*, allowing same-day reporting of negative results. Nevertheless positive results need to be confirmed by a sensitive and specific test to distinguish between toxigenic and nontoxigenic strains [6].

A rapid and easily-performed assay that has a high sensitivity and specificity is needed to reduce the morbidity from CDI and allow the implementation of infection control measures [7].

Aim of the study: was to introduce to our microbiology laboratory of a simple test that may be rapid, cheap and easily manipulated than conventional methods for effective diagnosis of CDI.

METHODOLOGY

Study design

This cross sectional study was carried out at the Clinical Pathology Department of Banha University Hospital over the period from August 2014 to July 2016. Sixty eight patients developing CDI like symptoms as watery diarrhea, fever, dehydration, abdominal pain, nausea, vomiting, loss of appetite, swollen abdomen, blood and/or mucous in stool were enrolled in the study. The patients were 40 males and 28 female, their ages ranged from 4 to 85 years with the mean age 37 ± 5.4 and they were classified into three groups; pediatric group ($4 \leq 18$) = 39, adult group ($>18 < 60$) =18, old age group (≥ 60) years = 11. Enrolling procedures comprised full medical history and clinical examination.

The study design was approved by the local Ethics Committee of Faculty of Medicine of Banha University

Laboratory methods

Stool samples were collected, transported immediately and subjected to the following:

Stool Analysis: was done according to the Standard Operating Procedures (SOPs) of **WHO** [8].

Isolation of C.difficile: by culture on *C.difficile* Agar Base (Oxoid, CM0601) supplemented with *C.difficile* selective supplement (Oxoid, SR0096) which is a fructose containing nutrient medium plus egg yolk, with D-cycloserine and cefoxitin as selective agents (CCFA). The medium was lightly inoculated with thioglycollate broth culture (Oxoid, CM0391), plates were incubated at 35°C for 48-72 hours in the Anaero Jar (Oxoid, AG0025A) with Anaerogen sachets (Oxoid, AN0025A)[9].

Identification of suspected colonies: it was done by colony morphology, characteristic odor, Gram stained film and simple latex agglutination test using *C. difficile* test Kit (Oxoid, DR1107) which is suitable for screening enrichment and selective cultures. Latex particles are coated with IgG antibodes specific for cell wall antigens of *C. difficile*. When the reagent mixed with a suspension of *C. difficile* colonies in saline on a reaction card, the latex particles agglutinate in large visible clumps within 2 minutes [10].

Detection of toxins by X/pect *C.difficile* toxin A/B test (Oxoid, R24650): is a qualitative immune-chromatography test that detects *C. difficile* toxin A and toxin B in stool samples or cultures of toxigenic *C. difficile*.

For direct X/pect test; stool sample was first diluted with specimen diluent, aliquot of the diluted sample was then mixed with equal volume of conjugate 1 containing antibodies to toxin A and toxin B coupled to colored micro-particles, plus a volume of conjugate 2 containing biotinylated antibodies. A 200 µl of this mixture was transferred to the circular sample well of the test device having immobilized streptavidin as a test line and goat anti-immunoglobulin antibody as a control line.

For toxigenic culture assays (indirect X/pect test); Brain Heart Infusion (BHI) broth (BIO-RAD, 64014) culture was made from suspected colony and incubated at 35°C for 72 hours in the Anaero Jar. 0.1 ml of broth culture was dispensed into a dilution tube then, five drops of conjugate 1 were added, followed by five drops of conjugate 2, mixed thoroughly and 0.2 ml was dispensed into the circular sample well of the test device. The test results were recorded visually after 20 minutes.

Immuno-complexes of toxin and conjugated antibodies form a visible band as they flow across the test area. Excess colored particles conjugates to form a visible band at the control area to ensure that the test was functioning appropriately [6].

Real time PCR for detection of *C. difficile* tcdA/ tcdB genes [11].

500 µl of stool sample and 1500 µl of S.T.A.R. Buffer (Roche Diagnostics) were added to 2.5-ml sterile Eppendorf tube, mixed, and centrifuged (4,000 × g for 1 min). 100 µl of the supernatant was transferred into a sterile Eppendorf tube together with proteinase K buffer (130 µl) and proteinase K (20 µl), followed by thorough mixing and incubation at 65°C for 10 min and then at 95°C for 10 min. 100 µl of the supernatant underwent automated DNA extraction on the MagNA Pure instrument (Roche Diagnostics).

For the tcdA assay, the following primers were used:
tcdA F, 5′GGTAATAATTCAAAAGCGGCT; tcdA R, 5′AGCATCCGTATTAGCAGGTG.

For the tcdB assay, the following primers were used: tcdB F, 5′GAAAGTCCAAGTTTACGCTCAAT; tcdB R, 5′GCTGCACCTAAACTTACACCA

The assay was performed on the Light-Cycler (Roche Diagnostics) with a total reaction volume of 20 µl (with 10 µl input DNA). Cycling parameters were as follows: program 1, 1 cycle of 50°C for 2 min; program 2, 1 cycle of 95°C for 15 min; program 3, 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s; program 4, hold for 4°C.

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 16 software (SpssInc, Chicago, ILL Company). Categorical data were presented as number and percentages. Z test for independent proportions (ZProp.) was used to analyze categorical variables, Cohen kappa test was used to assess degree of agreement and ROC curve was constructed to assess the performance of Xpect methods. The accepted level of significance in this work was stated at 0.05 (P <0.05 was considered significant).

RESULTS

Toxigenic *C. difficile* was detected in 15 out of 68 stool samples (22.1%) using real time PCR which was the gold standard in our study. The positive rate for the direct X/pect test was 13.2% (9/68 cases) and for the indirect test was 14.7% (10/68 cases).

Comparing with the real time PCR, the sensitivity of direct X/pect test was 60%, specificity was 100%, PPV was 100%, and NPV was 89.8% (table 1 , fig 1) and there was 91.2% agreement between both assays (kappa = 0.7, P < 0.001) as shown in table (2). While, the validity values for the indirect X/pect test was 66.7%, 100%, 100% & 91.4% for sensitivity, specificity, PP and NP values respectively, and there was 92.6% agreement between both assays (kappa = 0.757, P<0.001) for detection of CDI as shown in table (3). As regards, the direct and indirect X/pect test, there is 98.5% agreement between the results of the two methods (Kappa test = 0.939, P < 0.001).

Time to result for culture-based method (indirect method) was up to 3 days, while time to result for the immuno-chromatography test was less than 30 minutes when performed directly from stool samples.

CDI in our study was more common in males than females with a male to female ratio of (2:1) but this result was of no statistical significant value (P=0.96). The highest incidence occurred primarily in the age group above sixty (8/15, 53.3%, P < 0.001), followed by the pediatric age group (4/15, 26.7%, P =0.006) and lastly the adult group (3/15, 20%, P=0.52) as shown in table (4).

Different risk factors were analyzed and findings demonstrated that antibiotic intake, recent hospitalization, number of hospitalization days (> 30 days), use of proton pump inhibitors and colon disease were important risk factors for the development of CDI as shown in table (5). Penicillins and cephalosporins were the most frequently associated antibiotics with CDI positive cases (P<0.001), followed by clindamycin (P=0.033) and they were demonstrated to be significant risk factors.

Acute onset diarrhea was found in 93.3% of *C.difficile* positive cases and the majority of them had progressive course (85.7%) and watery, offensive stool. Fever and vomiting (66.7% for each) were the most common symptoms after diarrhea followed by nausea (60%), decreased activity and abdominal cramps (33.3% for each).

White blood cell counts, CRP, ESR, hemoglobin and serum albumin levels were analyzed. Leukocytosis (WBCs >11.000) was present in all *C.difficile* positive cases (100%) followed by elevated CRP (80%), prolonged ESR (66.7%) and anemia in (57.1%) but all are of no significant statistical value (P > 0.05).

Table 1: Diagnostic performance test for direct and indirect X/pect test

Variable	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%	AUC	95%CI	P
Xpect direct	60%	100%	100%	89.8%	91.2%	0.80	0.64-0.96	<0.001 (HS)
Xpect indirect	66.7%	100%	100%	91.4%	92.6%	0.833	0.68-0.98	<0.001 (HS)

Table 2: Agreement between real time PCR and Xpect direct test

			PCR		Total
			Negative	**Positive**	
Xpect direct fecal test	negative	Count	53	6	59
		% within PCR	100.0%	40.0%	86.8%
	positive	Count	0	9	9
		% within PCR	.0%	60.0%	13.2%
Total		Count	53	15	68
		% within PCR	100.0%	100.0%	100.0%

Kappa test= 0.7, P<0.001 (HS), degree of agreement=91.2%

Table 3: Agreement between real time PCR and Xpect indirect test

			PCR		Total
			negative	positive	
Xpect indirect fecal test	negative	Count	53	5	58
		% within PCR	100.0%	33.3%	85.3%
	positive	Count	0	10	10
		% within PCR	.0%	66.7%	14.7%
Total		Count	53	15	68
		% within PCR	100.0%	100.0%	100.0%

Kappa test= 0.757, P<0.001 (HS), degree of agreement=92.6%

Table 4: Comparison between C.difficile positive and negative cases regarding age and sex:

	C.difficile positive (N0=15)		C.difficile negative (N0=53)		$Z_{prop.}$ test	P
Age group (years)	**N0**	**%**	**N0**	**%**		
Up to 18	4	26.7%	35	66%	2.72	0.006 (S)
> 18 < 60	3	20%	15	28.3%	0.64	0.52 (NS)
≥ 60	8	53.3%	3	5.7%	4.43	<0.001 (HS)
Sex	**N0**	**%**	**N0**	**%**	X^2	**P**
Male	10	66.7%	35	66%	0.002	0.96 (NS)
Female	5	33.3%	18	34%		

Table 5: Frequency of different risk factors with *C.difficile* positive and negative cases

Risk factors	*C. difficile* positive (N0=15)		*C. difficile* negative (N0=53)		$Z_{prop.}$ test	OR (95%CI)	P
	N0	**%**	**N0**	**%**			
History of antibiotic intake	13	86.7	12	22.6	4.54	22.2 (4.4-112.4)	<0.001 (HS)
Recent hospitalization	13	86.7	18	33.96	3.62	12.6 (2.5-62.2)	<0.001 (HS)
number of hospitalization days (more than 30 days)	10	66.7	7	13.2	4.22	13.1 (3.5-49.9)	<0.001 (HS)
proton pump inhibiters (PPIs)	7	46.7	13	24.5	1.66	2.7 (0.82-8.8)	0.096 (NS)
Diseases of the colon	4	26.7	10	18.9	0.66	1.56 (0.41-5.9)	0.51 (NS)
Previous surgery of GIT tract	2	13.3	10	18.7	0.496	0.66 (0.13-3.4)	0.61 (NS)

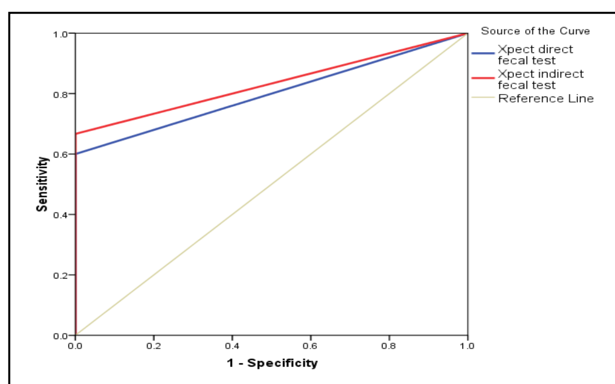

Fig. 1: ROC curve for direct and indirect X/pect test

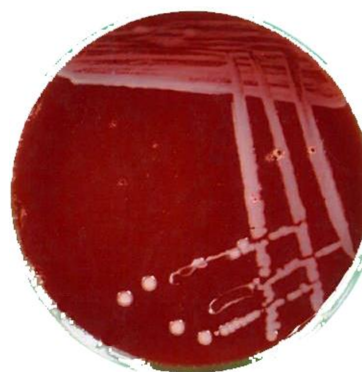

Fig. 2: *C.difficile* colony morphology on CCFA *medium*

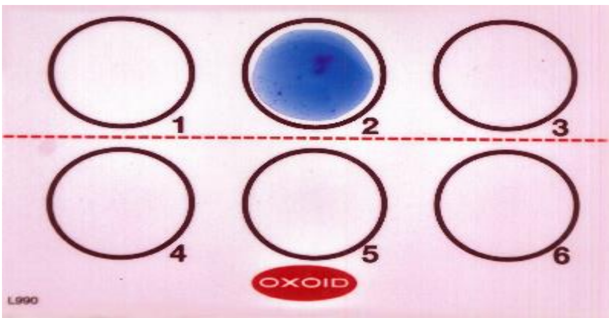

Fig. 3: Large visible clumps within 2 minutes indicates positive *C.difficile* latex test

Fig. 4: Positive X/pect *C. difficile* toxin A/B test

DISCUSSION

C. difficile infection is commonly responsible for pseudomembranous colitis (PMC) and antibiotic associated diarrhea, as well as post-operative diarrhea. The accurate identification of *C.difficile* provides important data for diagnosis, proper treatment and risk assessment studies[2].

There has been an increase in the occurrence and severity of CDI, partly due to the emergence of the hypervirulent strain BI/NAP1/027 that may be refractory to standard treatment and cause disease even in immune-competent individuals without prior antibiotic exposure and partly owing to the high rates of disease recurrence [12].

In the present study, toxigenic strains of *C. difficile* could be detected in (22.1%) (15/ 68) of tested stool samples by tcdA/ tcdB real time PCR. In accordance with our results, Morsi et al [13] reported an incidence of 21% for CDI at Zagazig University Hospitals using EIA and PCR methods during the period from April 2014 and May 2015. Also Miendje et al [14], Alcalá et al [15] & Shin et al [16] detected *C.difficile* in 23%, 27% and 26.3% respectively in samples from patients with clinical signs compatible with CDI.

Higher detection rate (46.2%) was reported by Abd El-Wahab et al [17] in a study conducted over one year period on 60 patients admitted at Tanta University Hospital using culture and ELISA techniques. On the other hand, in Texas Children's Hospital, Luna et al [18] found that 14% of tested samples was positive for *C. difficile* by EIA and in Canada, René et al [19] reported that *C. difficile* was isolated in (14.4%) of suspected cases.

Variability in detection rates can be partially explained by different sensitivities of the different diagnostic tests used; demographic characteristics of the patients such as age, sex; type of sample; selected or randomly collected, differences in antibiotic prescribing practices, infection control measures, or the occurrence of outbreaks during the study period [20].

The diagnostic performance of the X/pect test was done using tcdA/ tcdB real time PCR as the reference method and we found that the sensitivity of direct X/pect test was 60%, specificity was 100%, PPV was 100%, and NPV was 89.8% and there was 91.2% agreement between both assays (kappa = 0.7, $P < 0.001$). While, the validity values for the indirect X/pect test was 66.7%, 100%, 100% & 91.4% for sensitivity, specificity, PP and NP values respectively, and there was 92.6% agreement between both methods (kappa = 0.757, $P<0.001$) for detection of CDI. An evaluation report was produced by Eastwood et al., [21] and stated that X/pect test had sensitivity of 77.8%, specificity: 98.8%, PPV: 87.5, NPV: 97.6 and it had the highest specificity among the nine rapid tests evaluated in their study.

Lower validity values were reported by Sloan et al [22] who found the sensitivity of the X/pect test was 48% and the specificity was 84% and by Alcalá et al [15] who reported a sensitivity of: 49.0%, specificity: 95.8% PPV: 82.0% NPV: 83.0%. However, higher values were observed with Miendje et al [14] who reported a sensitivity of 91.3%, specificity: 100% , PPV: 100%, NPV: 97.5% and with Planche et al [23] who found the sensitivity was 82.0% and the specificity was 96.2%. This variation may be due to; the number of samples studied is very limited in most reports and the use of different gold standards in their studies, as the accuracy of the reference standard will have an impact on the performance of the rapid tests.

The assessment of a rapid test for detection of *C. difficile* can be affected by a number of factors including; how the test was administered, availability of trained laboratory personnel, differences in laboratory processing of samples and any modifications to the intended protocol. In addition the accuracy of the reference standard will have an impact on the performance of the rapid tests [3].

The low sensitivity and the high rate of false-negative X/pect toxin A/B test results in this study may be explained by several reasons Firstly; negative test result does not exclude the likelihood of the presence of *C.difficile* toxin A and/or toxin B. This may occur when the toxin level in the sample is beneath the detection limit of the test (A at level of ≥ 6.25 ng/ml (0.12 ng /test) and toxin B at level of ≥ 40.0 ng/ml (0.76 ng/ test) and the decision must be made by the physician in light of other laboratory results and clinical findings. Secondly; is the possibility of toxin degradation before performing the X/pect test as reported by René et al [19] Thirdly; is that the confirmed isolate by latex agglutination test might be non-toxigenic strains but only part of asymptomatic carriage in the GIT as it is estimated that 20% of hospitalized patients and up to 80% of healthy newborns and infants test positive for *C. difficile.*

Time to result for culture-based method was up to 3 days, while time to result for the immune-chromatography test was less than 30 minutes when performed directly on stool samples, allowing patients to be treated early and correctly in order to avoid complications and shorten the duration of symptoms.

The decision to use rapid test for the detection of *C. difficile* and the type of test used may depend on the cost of the test, the need for further equipment or training to do the test, the need for a short turn-around time to complete the test, the number of samples that are processed by the laboratory, the level of laboratory skills of the person performing the test, and the capacity to perform confirmatory tests by either cytotoxin assay, anaerobic culture or molecular methods. It was suggested that rapid tests could be used as a preliminary screening approach to identify potentially *C. difficile*-positive individuals. The use of a rapid test would decrease the delay in detecting a *C. difficile* infection and this may prove to be an appropriate option for those institutions that process samples on an occasional basis[23].

The highest incidence of CDI in our study occurred in the age group above sixty (53.3%), this is nearly similar to that found by Robin [24] in USA **&** Morsi et al [13] in Zagazig University Hospitals. This may be explained by increased needs of old age individuals to healthcare facilities, increased antibiotic usage, and impaired immune response to infectious pathogens.

CDI in this study was more common in males (66.7%) than females (33.3) with a ratio (2:1). In agreement with our results, Morsi et al [13] in Zagazig University Hospitals found that males (57.1%) were more affected than females (42.9%). Ophélie et al [25] in France also agree with our results, however Lucado et al [26] in the United States reported higher rates of CDI in females compared to males. Host factors related to the immune system may explain the differences in CDI rates between males and females [27].

Our findings demonstrated that recent hospitalization, antibiotic intake, hospitalization days (>30 days), the use of PPIs and colon disease were significant risk factors for the development of CDI and these results are in harmony with that of Ohshima et al [28]**.** Also in accordance with the present study, Leffler and Lamont [29] reported that recent hospitalization, antibiotic intake, use of gastric acid blockers, inflammatory bowel disease and previous gastrointestinal surgery were among risk factors for the development of CDI.

Many studies reported that antibiotic exposure is the most important risk factor, although single dose of antibiotics can cause CDI, greater number of antimicrobials used, greater number of doses, and longer duration of antibiotic administration increase the risk. It has been suggested that antibiotic administration may result in a disturbance in the normal gut flora and renders the individual liable to colonization by spores of *C. difficile*. These spores can be found in contaminated bed rails, toilets, and other surfaces inside hospitals and long-term care facilities [30].

In our study, penicillins and cephalosporins were the most commonly implicated antibiotics with CDI positive cases (33.3% for each, P <0.001) followed by clindamycin (20%, P=0.033) then aminoglycosides and carbapenems (6.6 % for each, P= 0.058 & P= 0.88 respectively). Abd El-Wahab et al [17] in Tanta University Hospital found that penicillins, cephalosporins and aminoglycosides were the most commonly implicated antibiotics with CDI. Moreover, Ohshima et al.[28] also demonstrated cephalosporins, carbapenems and fluoroquinolones to be risk factors.

The clinical presentation of CDI can range from asymptomatic carriage in the GIT, mild diarrhea, to potentially fatal PMC [31]. Acute onset diarrhea was found in 93.3% of *C.difficile* positive cases in our study and the majority of them had progressive course (85.7%) and watery, offensive stool. Fever and vomiting (66.7% for each) were the most frequent symptoms among positive cases after diarrhea followed by nausea (60%), decreased activity and abdominal cramps (33.3% for each). This finding was in accordance Abd El-Wahab et al [17] in Tanta and Morsi et al [13] in Zagazig, they reported that watery diarrhea was the most common symptom followed by abdominal pain, fever, nausea and vomiting, diarrhea was rarely bloody. In disagreement with our results Asem [32] in Jordan reported that about 50% of the infected populations had bloody diarrhea.

Leukocytosis (ranged from 11,800 to 14,900 per µl) was present in all C.*difficile* positive cases (100%) in our study, followed by elevated CRP (80%), elevated ESR (66.7%) and anemia in (57.1%)**.** This finding was in accordance with that of Williamson et al [33] who reported that WBC count < 15,000 per µl was present in all moderate CDI. On the other hand our results differ

from that of Asem[32] in Jordan who stated that leukocytosis is common but found in fewer than half of patients.

CONCLUSION

Culturing of *C. difficile* is time-consuming and labour intensive and a delay in the diagnosis could postpone treatment of a *C. difficile* positive individual. Using Xpect *C. difficile* toxin A/B test can combine accurate results with simple procedure that offers results within 20 minutes. Although it is associated with low sensitivity and high rate of false-negative results, X/pect test may be of great benefit to practitioners particularly when you need STAT testing or 24 hour/ 7 days coverage. Further, it can be used as a preliminary screening approach allowing patients to be treated early and correctly in order to shorten the duration of symptoms and avoid complications.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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