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The study compares two current methods, latex slide agglutination (LA) and enzyme immunoassay (EIA), used to detect ***clostridium difficile*** *- associated disease in the stool of suspected cases. Cytotoxic assay (CTA) was used as the gold standard. Of the 194 stool samples, there were 36 and 22 from male and female children, respectively. The remaining clinical fecal samples were from 60 male and 76 female adults. All current tests had a 9.76% positive value and 66.49% negative value.*

Using CTA test as the gold standard method, the LA test was shown to have 82% sensitivity; 94.73 specificity and an 18% false positive rate. Using the same gold standard method, the EIA test was shown to have 71.69% sensitivity; 91.93% specificity; and a 28.31% false positive rate. Although the EIA method is not time-consuming (about 3 hours), the test is rather less effective compared with the LA method. The advantage and disadvantages of all the current tests are also discussed in this article.

Key words : *Evaluation of Enzyme Immunoassay test and Latex Agglutination test,* ***Clostridium difficile.***

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นราทร ธรรมบุตร, ผดุงศรี วิชิวานิเวศน์, มยุรี ขันติพงศ์. คุณค่าการทดสอบอย่างเร่งด่วนด้วยวิธีเอ็นซัยม์ อิมมิวโน เอสเส เพื่อวินิจฉัยโรคลำไส้ใหญ่อักเสบ เนื่องจากแอนแอโรบิค คลอสตริเดียม ดิฟฟิซายด์. จุฬาลงกรณ์เวชสาร 2536 พฤษภาคม; 37(5) : 327 - 333

ได้ศึกษาเปรียบเทียบการทดสอบวิธีการต่าง ๆ ที่นิยมกัน 2 วิธี จากอุจจาระผู้ป่วยที่สงสัยว่าเป็นโรคติดเชื้อ เนื่องจาก ***คล็อสตริเดียม ดิฟฟิซายด์*** *โดยถือเอาการหาสารพิษชนิด cytotoxin ของแอนแอโรบิค ดังกล่าวเป็นมาตรฐาน (CTA) จากจำนวนอุจจาระผู้ป่วยเด็ก 58 ตัวอย่าง ซึ่งเป็นเด็กชาย 36 ตัวอย่าง เด็กหญิง 22 ตัวอย่าง นอกจากนั้นเป็นสิ่งส่งตรวจจากผู้ใหญ่ชาย จำนวน 60 ตัวอย่าง เป็นของสตรี 76 ตัวอย่าง ผลการศึกษาปรากฏว่า ทุกวิธีที่ทดสอบให้ผลบวกเหมือนกันคิดเป็นร้อยละ 9.76 และให้ผลลบร้อยละ 66.49*

วิธี Latex slide agglutination (LA) เมื่อเทียบกับวิธีมาตรฐาน CTA ได้ค่า sensitivity ร้อยละ 82, ค่า specificity ร้อยละ 94.73, ค่า false positive rate ร้อยละ 18

วิธี Enzyme immunoassay (EIA) เมื่อเทียบกับวิธีมาตรฐาน ได้ค่า sensitivity ร้อยละ 71.69 ค่า specificity ร้อยละ 91.93, ค่า false positive rate ร้อยละ 28.31

การศึกษาเปรียบเทียบชี้ให้เห็นว่า วิธี EIA นั้นแม้ว่าจะได้ผลเร็ว แต่ก็ด้อยกว่าวิธี LA อยู่ อนึ่ง คณะผู้วิจัย ฯ ได้วิจารณ์ข้อดี ข้อเสีย ของวิธีต่าง ๆ ที่นิยมในรายงานนี้ด้วย

Clostridium difficile is the major cause of diarrhoea among hospitalized patients. Its role in nosocomial antibiotics - associated diarrhoea (AAD) has been extensively studied and reviewed,[1-10] approximately 15-25% of AAD cases are caused by *Cl. difficile*.[7] McFarland et al. have remarked that if a patient has been hospitalized for more than two days and develops diarrhoea,[11] the first test should be for *Cl. difficile*. Parasites, shigella, salmonella are not recognized as common causes of nosocomial diarrhoea. *Cl. difficile* is the most prevalent single agent of nosocomial diarrhoea, especially following antibiotic therapy.[10,12] Carrier rates for *Cl. difficile* in hospitalized patients have been reported to be as high as 20%.[13] The majority of those habouring the organism remain asymtomatic, but in the patients who develop symptoms, the spectrum of disease ranges from self- limiting diarrhoea to acute colitis and life - treatening megacolon.

Most strains of *Cl. difficile* produce both an enterotoxin (toxin A or D1) and a cytotoxin (toxin B or D2). Toxin A has been linked with disease and may therefore be the most important marker for disease in humans. Toxin B (D2) is a potent *in vitro* cytotoxic agent, with little biological activity *in vivo*.[14,15]

Diagnostic testing in cases of suspected *Cl. difficile* - associated diarrhoea can be accomplished by recovering the organism in culture, by demonstrating the presence of toxin B in stool by a tissue culture assay, or by performing a rapid latex agglutination test for *Cl. difficile* - associated antigen on stool specimens. These tests can be used alone or in combination.[5,6]

In early 1985, *Cl. sordellii* antitoxin (Lot 40067-3666) was donated to our Anaerobic Division by the United States Food and Drug Administration (FDA), Bethesda, Maryland; since that time the Division has been operating successfully and routinely carrying out tissue culture assay for *Clostridium difficile* cytotoxin.[3,4] In 1987, the simple- rapid latex slide agglutination test has been tried[16] for the identification of *Cl. difficile* antigen from cases of suspected *Cl. difficile* - associated disease.

In early 1992, the use of the new rapid premier enzyme immunoassay (EIA) test * was introduced by our Anaerobic Division. This specific test detects *Cl. difficile* toxin A in stool specimens for the diagnosis of *Cl. difficile* - associated diseases (CDAD).[15] In order to evaluate the advantages and disadvantages of the new rapid EIA test, other current tests should also be done together with the afore mentioned rapid EIA test.

Materials and methods

Fecal samples : A total of 194 stool specimens were obtained from patients at Chulalongkorn Hospital Medical School, Bumrungraj General Hospital, Bangkok Christian General Hospital, Samithivej General Hospital and Kluaynamthai General Hospital. The patients were compromised hosts with a history of either recent (within 8 weeks) antibiotic treatment or about three loose stools per day for at least two days. The clinical samples were submitted to the Anaerobic Laboratory, Department of Medical Microbiology. ** Immediately after receipt (normally within two hours after collection), the samples were processed as follows :

1. Rapid EIA test for *Cl. difficile* -toxin A A. Rapid EIA test: the premier *Cl. difficile* toxin A test is a rapid (2 1/4 hrs.) microwell- based EIA which detects the presence of toxin A in human stool specimens.

a. Specimen processing: Stool specimens (50 ml each) were throughly mixed with 200 ml of a given sample diluent, + with the suspension Gently expelled and withdraw several times, and vortexed for 15 seconds.

b. Sample and enzyme conjugate incubation : The microwells * needed were detached, placed in holder with one drop of enzyme conjugate ** added to all wells (including those containing positive and negative controls). Then one drop of the diluted stool was added to the appropiate microwell; one drop of a positive or negative control was added to the appropiate well, shaken firmly for 30 seconds, sealed and incubated at 37 ^{0} C for two hours.

c. Substrate incubation : The wells were emptied and carefully washed five times with the given wash buffer.

One drop of substrate A with chromogen indicator was added to each well, and later one drop of substrate B with chromogen was also to each well.

The mixture was shaken firmly for 30 seconds and incubated for 10 minutes at room temperature. One drop of stop solution (sulfuric acid) was added to each well and shaken firmly for 30 seconds. They were read for color development within 30 minutes.

d. Interpretation of result (visual appearance regulation);

Colorless = neagative ; faint yellow color= indeterminant; and definite yellow color= positive.

B. Cytotoxic assay (CTA) directly from the stool filtrate for *Cl. difficile* biological toxin.

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* From Meridian Diagnostics, Cincinati, Ohio 45244, U.S.A.

** Faculty of Medicine, Chulalongkorn University, Bangkok.

* Sample diluent-protein solution with a preservative.

* Using Bio-Tek El 310. Bio Tek Instrument, Inc., Winooski, VT, U.S.A.

** Antibody to toxin A conjugated with monoclonal horse radish peroxidase.

C. Conventional culture (CUL) for *Cl. difficile* and determination of toxin production *in vitro* by the cytotoxicity assay.

D. Latex slide agglutination test (LA) directly from the stool specimen.

The method of isolation of *Cl. difficile* from clinical samples, the cytotoxic assay (CTA) and the latex slide agglutination test (LA) were described by Dhamabutra N et al. [15]

Results

A total of 194 stool specimens collected from 194 patients were included in this study. These fecal samples were from 58 children and 136 adults with suspected CDAD * (Table 1).

Table 1. Age distribution of of 194 patients evaluated for *Cl. difficile* associated colitis.

Month	Clinical fecal specimens		Age-distribution: Children (0-10 years)		Age-distribution: Adults (11-60 years)	
	Total specimen (%)		Males	Females	Males	Females
Jan	27	(13.17)	4	2	12	9
Feb	38	(19.58)	11	5	12	10
Mar	34	(17.52)	9	4	9	12
Apr	30	(19.46)	5	5	8	12
May	28	(14.43)	3	4	8	13
Jun	37	(19.0)	4	2	11	20
Total number of specimens	**194**		**36**	**22**	**60**	**76**

Each specimen was tested using four laboratory methods for the detection of *Cl. difficile* : namely, EIA, isolation of *Cl. difficile* (CUL), a tissue culture cytotoxicity assay (CTA) and latex slide agglutination test (LA). [12] A comparison of these methods showed that each clinical sample from the suspected eases of CDAD produced either one or more positive tests. Out of 194 samples, 129 samples were negative by all tests.

CTA is the gold standard method; however, in the 38 EIA positive samples, all CTA tests were all negative. Compared with two showing positive results by the CTA method, the EIA test revealed 17 negative samples. The LA test revealed 54 positive samples while there were 41 positive with the CTA method. The LA revealed 11 negative, samples compared with 9 positive by the CTA test (Table 2.)

Table 2. Comparison of enzyme immunoassay (EIA), latex agglutination (LA) with cytotoxicity assay (CTA) from the most likely cases of suspected CDAD.

Test method	Results: Positive	Results: Negative	CTA test (Gold standard): Positive	CTA test (Gold standard): Negative
EIA	38	0	38	0
EIA	0	17	15	2
LA	54	0	41	13
LA	0	11	9	2

* CDAD = *Cl. difficile* - associated disease.

The sensitivity and the specificity of the EIA test was 71.69% and 91.93%, respectively. (Tables 3,4). The sensitivity and the specificity of the LA test was 82% and 92.73%, respectively (Table 5,6).

Table 3. Results of *cytotoxic* assay (CTA) and enzyme immuno assay (EIA) of *Cl. difficile.*

Method of identification	Positive fecal samples	Negative fecal samples	Total number of samples	Percentage of positive fecal specimens
CTA	38	156	194	19.58
EIA	23 * (38-15)	171	194	11.85

* Refers to Table 2: the number of real positive fecal samples as compared with those identified by the gold standard method (CTA).

Table 4. Evaluation of *Cytotoxic assay* (CTA) with enzyme immunoassay (EIA) of *Cl. difficile.* *

Result of Cl. difficle detection	Positive fecal samples	Negative fecal samples	Total number
Positive	38	15(38-23)	53
Negative	15(38-23)	171(EIA)	186
Total	53	186	

Sensitivity	= 71.69%
Specificity	= 91.93%
Positive predictive value	= 71.69%
Negative predictive value	= 91.93%
False positive rate	= 28.31%

* Calculation base on Chumni-jarlkit T. Applied Medical Statistics,2 nd ed. Bangkok. Chulalongkorn University Publication 1984 : 50-51.

Table 5. Results of cytotoxic assay (CTA) and latex agglutination test (LA)of *Cl. difficile.*

Method of Cl. difficile identification	Positive fecal samples	Negative fecal samples	Total number of samples	Percentage of positive fecal specimens
CTA	41	153	194	21.13
LA	32 * (41-9)	162	194	16.49

* Referred from Table 2, the real No. of positive fecal sample as compared to the gold standard method. (CTA)

Table 6. Evaluation of cytotoxic assay (CTA) compared with latex agglutination test (LA). *

Result of Cl. difficile detection	Positive fecal samples	Negative fecal samples	Total fecal samples
Positive	41	9(41-32)	50
Negative	9(41-32)	162(LA)	171
Total	50	171	
Sensitivity		= 82%	
Specificity		= 94.73%	
Positive predictive value		= 82%	
Negative predicitive value		= 94.73%	
Falše positive rate		= 18%	

* Calculation base on Chumni-jarlkit T. Applied Medical statistic. 2 nd ed. Bangkok. Chula Univ Publication 1984 : 50-51

Table 7. Comparison of culture (CULT,) cytotoxic assay (CTA), latex agglutination (LA) and enzyme immunoassay (EIA) from the most likely cases of CDAD. *

Number of stool samples	CULT	CTA	LA	EIA	%
19	+	+	+	+	9.76
18	-	+	+	+	9.28
8	+	-	+	-	4.12
2	+	-	-	-	1.03
8	-	+	-	-	4.12
5	-	-	+	-	2.58
4	+	+	+	-	2.06
1	-	+	-	+	0.52
Totol 129	ï	ï	ï	ï	**66.49**

* CDAD = *Cl. difficile* - associated disease.

Discussion

The diagnosis of CDAD remains problematic. The presence of diarrhoea with a history of recent concomitant antimicrobial therapy is only suggestive of disease. Laboratory tests must be used to aid the diagnosis.

Isolation of *Cl. difficile* on CCFA has also been used. The method has been shown to be more sensitive than, but not as specific as, cytotoxin testing. [3,4,12,17]

However, false positive as well as false negative cytotoxin assays are also possible. In previous studies, culture was shown to be the most sensitive and least specific test for CDAD, whereas the cytotoxin assay was the least sensitive and most specific test for the disease. [3,4,12,17]

According to Table 1, male children were suspected of having CDAD morefrequenthy than females, whereas frequency among female adults was higher than among male adults. Thus, CDAD affiliate both sexes and all age groups.

In Table 7, all clinical specimens were tested by all methods (CTA, LA and EIA); conventional identification (CULT)
for *Cl. difficile* was also performed.

The percentage positive by all tests was only 9.76%, which shows the different significant susceptibility and specificity of each test. However CTA was considered as the gold standard test because isolation and culture are meaningless unless one can prove that the anaerobes harbour the toxin. [3] Whenone establishes CTA as the gold standard, the LA test reveals more positive cases than the EIA method. The LA test is more suitable as a screening test than the EIA method (Table 2).

The EIA test has less specificity and sensitivity than the LA method because toxin A may lose potency in the stool specimens during the process of specimen collection and fecal extraction (Tables 3,4,5 and 6). Moreover, with the EIA method, we did not use the spectophotometer to read the final result.

With regard to time, the EIA test requires only 2 1/ 2 hours to conduct, while CTA requires at least 48 hours and LA about 24 hours. Therefore, the rapid EIA test shoud be vsttirf out in combination with the LA test is order to diagnoses suspected cases of CDAD.

The EIA test demonstrated lower sensitivity because the test was negative for one out of fours positive specimens which were positive for all other laboratory tests (Table 7).

EIA demonstrates good specificity and good sensitivity compared with cytotoxin assay. Although LA is not as rapid as EIA, the test can be performed in several hours, and is suitable as a screening test in Thailand. EIA can be used alone or in combination with other methods to provide rapid and sensitive results.

Recent articles have discussed the need for a rapid test for toxin A. The *Cl. difficile* toxin A EIA presents an important bridge method in the field of *Cl. difficile* testing. The test is rapid (and yet possesses a high level of sensitivity and specificity comparable to the more time - consuming cytotoxin assay.

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