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Evaluation of current methods for laboratory diagnosis of anaerobic Clostridium difficile-colitis*

Narathorn Dhamabutra*
Padungsri Vichivanives*

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A study of 72 infected fecal specimens of suspected Clostridium difficile - colitis was made using various current methods for laboratory diagnosis.

Primarily, the latex slide agglutination of the suspected Cl.difficile colonies were compared to the classical isolation with identification by fermentative and biochemical method. The is method yielded 19.4 sensitivity; 78.57% positive predictive value; 21.43% false positive while the latter method gave only 15.27% sensitivity.

Secondly, the indirect detection of Cl. difficile cytotoxin of Cl. difficile colonies were compared to the gold standard cytotoxicity bioassay detection of Cl. difficile - toxin, collected directly from the infected stool samples. The indirect-method of Cl. difficile toxin detection revealed 12.5% sensitivity; 55.55% positive predictive value; 44.45% false positive rate while the gold standard method yielded 6.5% sensitivity only. The practical and up to date laboratory method suitable for use in Thailand is also discussed in this article.

Reprint request : Dhamabutra N, Department of Microbiology, Faculty of Medicine, chulalongkorn University, Bangkok 10330, Thailand.
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* Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

นราทร ธรรมบุตร, ผดุงศรี วิชิวานิเวศน์. การศึกษาคุณค่าวิธีการทดสอบต่าง ๆ เพื่อวินิจฉัยโรคลำไส้ใหญ่อักเสบจากแอนแอโรบบิค คลอสตริเดียม ดิฟฟิซายล์. จุฬาลงกรณ์เวชสาร 2532 มิถุนายน ; 33(6): 419-427

ได้ศึกษาถึงวิธีการต่าง ๆ ในการวินิจฉัยโรคทางห้องปฏิบัติการ สำหรับโรคลำไส้อักเสบ โดยการตรวจอุจจาระของผู้ป่วยจำนวน 72 สปีศิเมนส์ วิธีการทดสอบทางห้องปฏิบัติการนั้น มี 2 ประเภท ประเภทแรก คือแยกวิเคราะห์หาแอนแอโรบิค คล๊อสตริเดียม ดิฟฟีซายล์ จากอุจจาระซึ่งปรากฏว่า การใช้วิธี Latex slide agglutination ให้ผลความไวถึงร้อยละ 19.4; มีค่า positive predictive ร้อยละ 78.57; false positive rate ร้อยละ 21.43: ซึ่งวิธีมาตรฐาน fermentation นั้นมีความไวเพียงร้อยละ 15.27; ประเภทที่ 2 การหาค่าท็อกซินของคล๊อสตริเดียม ดิฟฟีซายล์ในอุจจาระ ปรากฏว่า การใช้วิธี cytotoxicity-bioassay จากโคโลนี คล๊อสตริเดียม ดิฟฟีซายล์ที่แยกได้ ให้ผลความไวร้อยละ 12.5; การหาท็อกซินของแอนแอโรบส์นี้จากอุจจาระพบว่าค่า positive predictive ร้อยละ 55.55; และค่า false positive rate ร้อยละ 44.45; ซึ่งด้อยกว่าวิธีหาท็อกซินโดยทางอ้อม จากโคโลนี แอนแอโรบส์ที่แยกได้

ผู้ป่วยที่สงสัยว่าเป็นโรคลำไส้ใหญ่อักเสบเนื่องจากแอนแอโรบส์นี้ มักจะเป็นผู้ป่วยที่มีภูมิต้านทานบกพร่องและจำเป็นต้องให้ยาต้านจุลชีพประจำและนาน งานวิจัยนี้ยังวิจารณ์วิธีการทดสอบทางห้องปฏิบัติการที่เหมาะสมกับประเทศไทยด้วย

Clostridium difficile was primary described by Hall and O' Toole as the normal flora of newborn infant in 1935.[1] This obligately anaerobic, Gram-positive rod with oval subterminal spore, is now firmly established as the principal causative agent of pseudo-membranous colitis (P M C). Moreover, few workers would dispute the assertion that the organism is a major cause of a spectrum of antibiotic associated conditions ranging from florid colitis to mild, self-limiting diarrhoea. It has also become apparent that outbreak of diarrhoeae may occur as a result of cross-infection with *Cl.difficile.*[2]

The presence of *Cl.difficile* in fecal material may be established by the demonstration of the organism or its extracellular products. *Cl.difficile* is now known to produce two protein exotoxins.[3] The cytotoxin produced by most strains was designated toxin B (D-2) and the entero-toxin, toxin A(D-1). Both toxins exhibit cytotoxic activity, but that of toxin B is much greater than that of toxin A. Cytotoxin is present in the stools of most cases of histologically confirmed P M C, and there is a highly significant association between the presence of cytotoxin and *Cl.difficile* in the stools of patients with colitis[4]

The most frequently used method of detecting cytotoxin is the tissue culture assay, in which the cytotoxin, present in either a fecal extract or culture supernatant, is neutralized by *Cl.sordellii* antitoxin[5]

Following the purification of toxins A and B, several techniques have been applied to their detection using specific antitoxins. A latex agglutination test using antitoxin raised against purified toxin A was developed by Banno et al. Other latex agglutination tests have been described for deection of either toxin B or cellular antigens of *Cl.difficile.*[3,6] Latex agglutination assays for other enterotoxins are available commercially.

Since early 1985, *Cl.sordellii* antitoxin Lot 40067-3666* were donated by Dr.L E Bennett ** to our Anaerobic Division which has been operating successfully and routinely the tissue culture assay for *Clostridium difficile* cytotoxin.[7-9] In 1987, the simple-rapid latex slide agglutination test for the identification of *Cl.difficile* from the suspected colitis-patients' stool have been tried. The purpose of the study is to evaluate the advantage and disadvantage of laboratory methods for detection of either *Clostridium difficile* colonies or the cytotoxin in the patients' stool samples.

Materials and Methods

Fecal samples : A total of 72 stool specimens were obtained from patients at the Chulalongkorn Hospital Medical School. Most of the patients were compromised hosts and suspected of having pseudomembranous colitis. The clinical samples were submitted to the Anaerobic Laboratory, Dept. of Medical Microbiology*** for isolation of *Cl.difficile* and for toxin detection. Immediately after receipt (normally within 2 hrs. after collection), the samples were processed for culture and toxin detection.

Culture : Isolation of *Cl.difficile* was performed by the method of Koransky et al.[10] Approximately 1 gm. of undiluted stool specimen was emulsified in equal volume of 50% alcohol by vigorous mixing in a vortex mixer. The mixture was left at room temperature for 10 min. A few drops of the supernatant were cultured on cefoxitin-cycloserine fructose agar plates (C C F A), which were incubated anaerobically at 37°C.[11] These isolates were conventionally identified by fermentation and biochemical tests: The isolated suspected colonies were identified for species by fermentation and biochemical tests according to the method of Virginia Polytechnic Institute Anaerobe Laboratory Manual.[12]

Latex agglutination test : The Serobact *Cl. difficile* latex slide agglutination kit was supplied by Disposable Products, Adelaide, South Australia. Each kit contained latex particles that had been coated with an immunoglobulin G specific *Cl.difficile* cell wall antigens and a suspension of *Cl.difficile* to be used as a positive control. The test was carried out on black cardboard tiles that were supplied with the kit. Fresh subcultures of *Cl.difficile* on C C F A were used for assessment. When testing colonies from solid media a smooth suspension of organism was made in one drop (about 0.02 ml.) of saline and observed for autoagglutination, One drop of *Cl. difficile* latex reagent was added to this suspension. After mixing the slide was gently rocked and observed for agglutination up to 2 minutes before discarding. (Fig. 1,3)

The isolated colonies of *Cl.difficile* were subcultured in chopped-meat broth (Oxoid Ltd., England) and toxin in the culture supernatant was assayed by the cytotoxicity test (Fig. 2)

A portion of each fresh specimen was diluted with an equal volume of phosphate-buffered saline**** and centrifuged at low speed for 10 min. The supernatant was passed through a filter (pore size 0.45 um.) and the filtrate was assayed for toxicity (Fig. 2).

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* Undiluted *Cl.sordellii* antitoxin has been tested by Dr. E L Barbara of Food and Drug Administration (F D A), Bethesda U S A.

** Director, Division of Product Quality control, Center for Drugs and Biologics, F D A.

*** Faculty of Medicine, Chulalongkorn University.

**** Containing penicillin 1000 mg./1., streptomycin (1000 mg./1) and metronidazole (100 mg./1.)

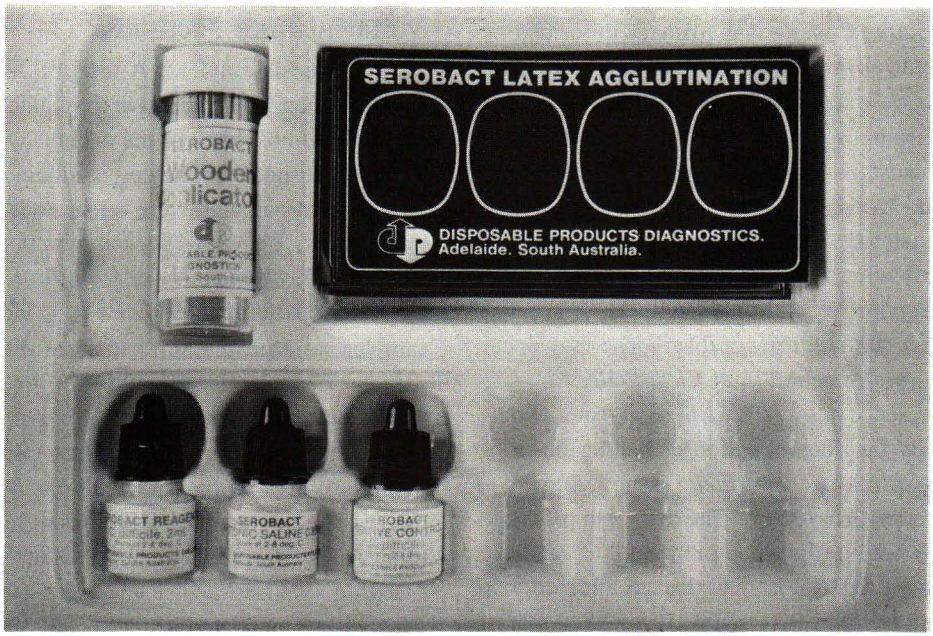
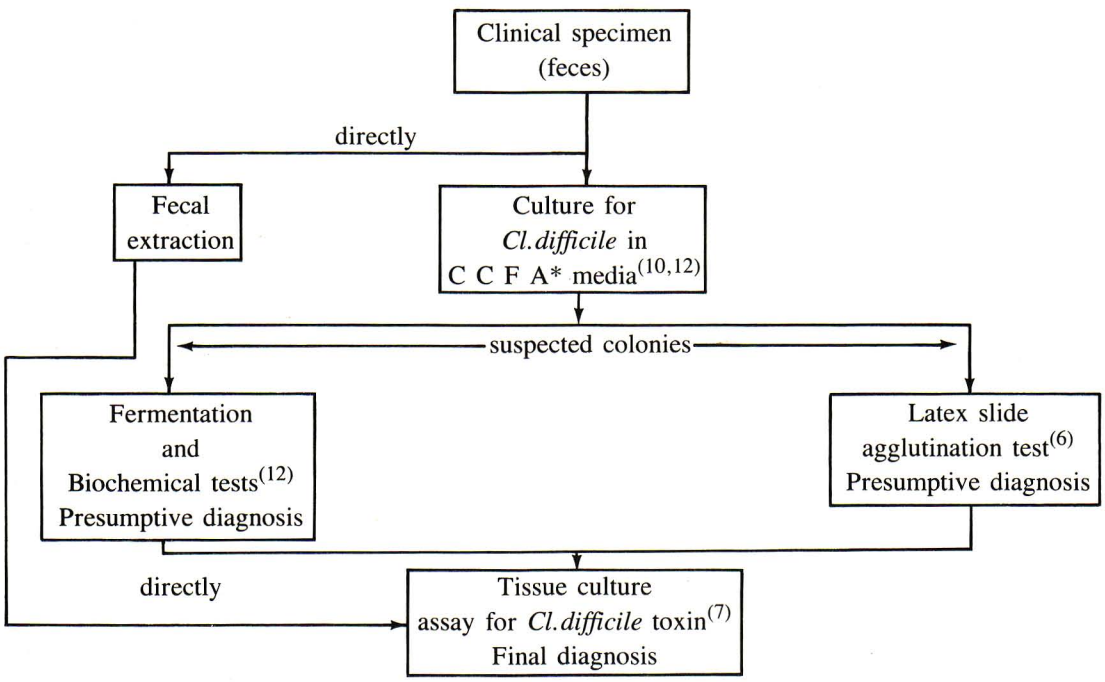


Figure 1. Showing Latex slide agglutination kit for the identification of *Cl.difficile.*

Figure 2. Diagram showing the methods for Laboratory diagnosis of *Cl.difficile* colitis.



* cefoxitin cycloserine fructose agar.

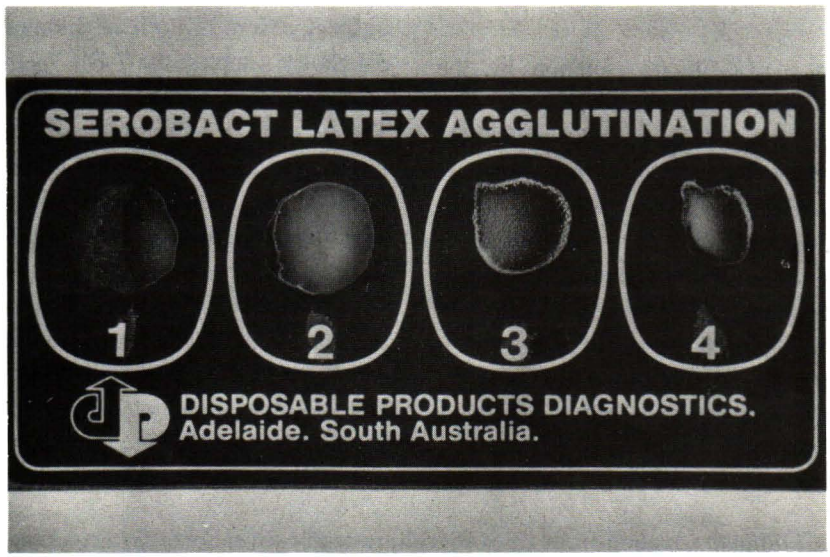
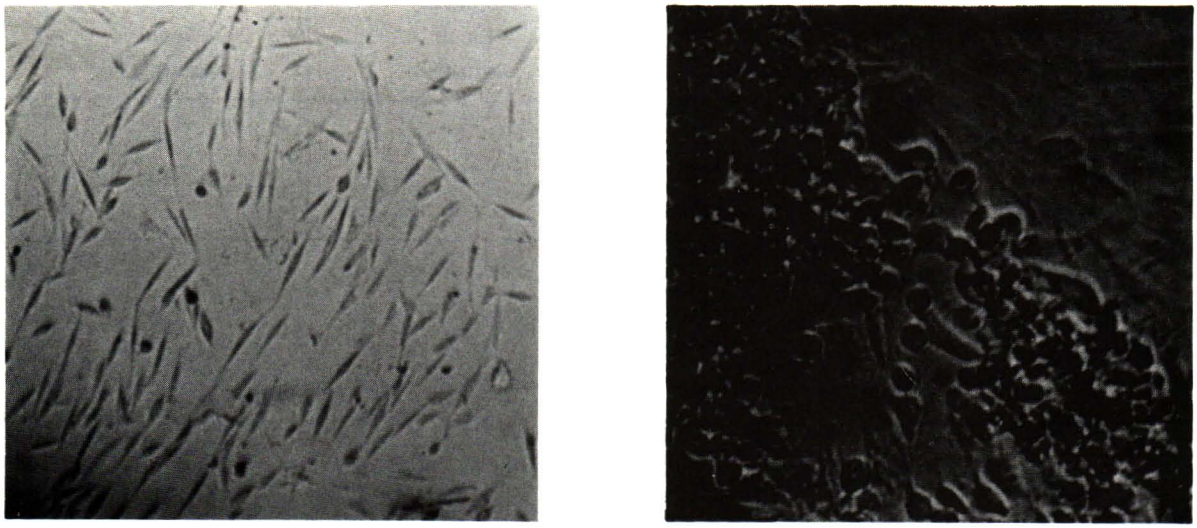


Figure 3. Showing the positive result of *Cl.difficile* by Serobact Agglutination on Serobact-slide.
1 Saline + suspected *Cl.difficile* reagent (No autoagglutination).
2 Saline + *Cl.difficile* reagent (No autoagglutination).
3 Control suspension + *Cl.difficile* reagent (positive).
4 Saline + suspected *Cl.difficile* colonies + *Cl.difficile* reagent (positive).

Cytotoxicity assay for *Cl.difficile* toxin : Either the culture supernatant or the fecal filtrate through the 0.45 um. filter was centrifuged 2000 g for 10 min. Each of the supernatant was removed and 4 drops (100 ul) added to each of two tubes containing a tissue culture monolayer (human foreskin fibroblasts)* *Cl.sordellii* antitoxin** (100 ul) was then added to one tube. A characteristic cytopathic effect neutralized by the anti-toxin after 24-48 hrs. incubation was regarded as a positive result[7] (Fig 2,4).

The primary human fibroblast cells were prepared from the foreskin tissues of one day old newborn infant. This newborn foreskin tissues obtained from circumcision was placed in the maintenance medium and kept cold while being delivering to the laboratory. The tissues submitted to the laboratory for primary cell culture should be processed as soon as possible[13] (Fig. 4).



(L) (R)

Figure 4. Fibroblast in normal morphology (L) and after cytopathic effect (R).

* The primary human fibroblasts were prepared from the foreskin tissues of one day old newborn.

** With the compliment of Drs. L E Bennett and E L Barbara.

Results

Comparison of *Cl.difficile* isolation by the original fermentation with biochemical test (C F B) with the latex agglutination method (C L A) is shown in Table 1 and 2. The C F B method had lower percentage of positive *Cl.difficile* colonies than the C L A method. However C L A method showed 3 false positive specimens. The positive predictive value of C L A method was 78.57%. The false positive rate in the C L A method was 21.73%. According to the conventional C F B method, C L A had 100% sensitivity and 95% specificity.

Table 3 and 4 evaluated the benefit of *Cl.difficile* toxin-detection either directly from feces (T D F) or indirectly from *Cl.difficile* colonies (T D C). Out of 72 diarrhoeal samples, the T D F method had only 55.55% sensitivity. The specificity of T D F method was 94.03%. The percentage of positive predictive value of T D P method was 55.55%. The T D F method had 14.45% false positive rate. All the tests in Tables 1 and 3 were performed in duplicate and no discrepant results were found when repeated.

Table 5 revealed the incidence of clinical diagnosis and the final cytotoxicity assay-results for 1987. Most cases had no clinical diagnosis on the request-sheet. Out of 72 fecal samples, 9 specimens were found to habour specific *Cl.difficile* toxin (Table 5).

Table 1 Result of *Cl.difficile*-isolation from feces by culture with conventional fermentation and biochemical test (C F B) and culture with latex slide agglutination method (C L A).

Methods of identification	Result of identification		Total fecal specimens	Percentage of positive fecal specimens
	positive fecal samples	negative fecal samples		
1. C F B (standard method)	11	61	72	15.27
2. C L A	14	58	72	19.40

Table 2 Evaluation of *Cl.difficile*-isolation from feces by culture with conventional fermentation and biochemical method (C F B) to culture with latex slide agglutination method (C L A).

Result of *Cl.difficile* identification	Positive fecal samples	Negative fecal samples	Total fecal samples
positive	11	3	14
negative	0	58	58
Total	11	61	72

Result of evaluation;

Sensitivity $= \frac{11}{11} \times 100 = 100\ \%$

Specificity $= \frac{58}{61} \times 100 = 95\ \%$

Positive predictive value $= \frac{11}{14} \times 100 = 78.57\ \%$

Negative predictive value $= \frac{58}{58} \times 100 = 100\ \%$

False positive rate $= 21.43\ \%$

Table 3 Result of the *Cl.difficile*-toxin detection directly from feces (T D F) and indirect toxin detection from the isolated colonies (T D C).

Methods of identification	Result of identification		Total fecal specimens	Percentage of positive fecal specimens
	positive fecal samples	negative fecal samples		
1. T D F	5	67	72	6.74
2. T D C (standard method)	9	63	72	12.5

Table 4 Evaluation of *Cl.difficile*-toxin detection directly from feces (T D F) compared to the conventional indirect toxin detection from the isolated colonies.

Result of *Cl.difficile* toxin detection	Positive fecal samples	Negative fecal samples	Total
positive	5	4	9
negative	4	63	67
Total	9	67	

Result of evaluation;

Sensitivity $= \frac{5}{9} \times 100 = 55.55\ \%$

Specificity $= \frac{63}{67} \times 100 = 94.029\ \%$

Positive predictive value $= \frac{5}{9} \times 100 = 55.55\ \%$

Negative predictive value $= \frac{63}{67} \times 100 = 94.03\ \%$

False positive rate $= 44.44\ \%$

Discussion

Clostridium difficile has been shown to be the causative agent of pseudomembranous colitis, diarrhoeae associated with antibiotics, non-antibiotics induced colitis chronic inflammatory bowel disease and postoperative diarrhoeae.[1,14-16] Several recent papers have described the detection of fecal cytotoxin and the latex slide agglutination test for the suspected colonial isolates.[6]

Compared to the original C F B method, Table 2 reveals that C L A method has a sensitivity of 100% and a positive predictive value of 78.57%. Moreover one can perform the C L A easily, rapidly and less expensively. Howover, the disadvantage of the C L A method is that several other *Clostridia* spp. may also give positive results.[6] This is the reason why C L A method has 21.43% false positive rate. Although C F B method* is the conventional, originally reliable test, it is time consuming and requires skillful-personels. Moreover, in the procedure of *Cl.difficile* isolation, the intestinal bacterial flora interfere with the specific isolation. Therefore the modified media of selective G C C** broth as described by Carrol et al should be tried with the help of Gas-liquid chromatography.[17] However, *Cl.difficile* may be found in really few clinical stool-samples. In view of this, the C L A method is more superior and rapid to

* Gold standard method.

** G C C broth, the selective media containing gentamicin, cycloserine and cefoxitin.[17]

Table 5 The result of cytotoxicity assay and the patients' clinical diagnosis (Year 1987).

Clinical diagnosis	No. of cases	Sexes				Result of Cytotoxicity assay	
		Adult		Children			
		Male	Female	Male	Female	Positive	Negative
1. Acute lymphoid leukemia	5	3	2	-	-	1	4
2. Acute non lymphoblastic anaemia	5	4	1	-	-	-	5
3. Acute myeloid leukemia	2	1	1	-	-	1	1
4. Acute pancreatitis	1	1	-	-	-	-	1
5. Cirrhosis-liver	3	2	1	-	-	-	3
6. Diabetes mellitus	5	3	2	-	-	2	3
7. Hepatic encephalopathy	1	-	1	-	-	-	1
8. Meningioma	4	-	4	-	-	-	4
9. Mitral stenosis	1	-	-	1	-	-	1
10. No clinical diagnosis	38	10	19	5	4	4	34
11. Post operative nephrectomy with tissue resection	3	-	-	3	-	-	3
12. Shigellosis	1	-	-	1	-	-	1
13. Subdural hematoma	2	1	1	-	-	1	1
14. Tuberculosis	1	1	-	-	-	1	-
TOTAL	72	26	32	10	4	10	62
Percentage	100	36.11	44.44	13.88	5.5	13.88	86.11

perform than the C F B method, and should therefore be more useful in a screening test.

The detection of *Cl.difficile* toxin from the patient's stool (T D F) is less sensitive than the indirect method (T D C) and therefore T D F has higher percentage of false positive rate (Table 3 and 4). Moreover, the percentage of sensitivity in T D F method is only 55.55%. This is because the normal intestinal fluid habours substantial kinds of toxic - substances either from its own microbial flora or from exogenous origin and in the disease-status, the patient's stool habours more and more abnormal state of microbial flora; therefore the *Cl.difficile* toxin in the fecal sample is interfered by influenced by many factors in the fecal sample.

Most patients stools were watery after long term antibiotic treatment. These patients were compromised hosts with immunodefficiency conditions and the specific current antibiotics were necessary for them. The long term antibiotic regimens certainly interfered with the colonic microbial flora when antibiotic associated colitis of *Cl.difficile* origin become suspected (Table 5).

In Thailand, the incidence of proved *Cl.difficile* colitis is rarely detectable.[5] The C L A* method has now been simplified considerably by replacing the C F B** method, enabling small local laboratories to improve their *Cl.difficile* isolation rate because *Cl.difficile* may persist in stool specimens for some time after cytotoxin is no longer detectable.[18] We therefore would like to recommend the C L A method as a useful screening test in the local community. The positive *Cl.difficile* pure culture from fecal specimens may then be sent to a larger laboratory where the facilities for tissue culture are available.

The *Cl.difficile*-colitis occurs at any age and sex but usually the patient has immuno defficiency and chronic underlying disease. In our study, most clinical fecal samples were from adults. For infants, *Cl.difficile* isolation is not helpful because this anaerobe is the normal intestinal flora.[1] Final Cytotoxicity bioassay is very significant (Table 5).

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* Culture with latex agglutination test.

** Culture with fermentation and biochemical test.

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