

## **Toxin A detection on *Clostridium difficile* colonies from 24-h cultures**

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**Objective:** Performance of a combined approach for the detection of toxigenic strains in patients suspected of having *Clostridium difficile*-associated disease was evaluated.

**Methods:** In this approach, stools were cultured for 24 h on a selective medium supplemented with sodium taurocholate (TCCFA), in anaerobic conditions created with the Mart<sup>®</sup> Anoxomat system, and toxin A detection was performed directly on *C. difficile* colonies, by enzyme immunoassay (EIA). This method was compared with three others: cytotoxigenic culture consisting of a 48-h culture on selective medium followed by detection of in vitro cytotoxin production on cell monolayers, fecal cytotoxin detection and fecal toxin A detection by EIA.

**Results:** From 548 stools, 108 yielded a positive culture by at least one of the methods, and 81 isolates were cytotoxin producers. Cultures for 24 h on TCCFA were positive in 106 cases and EIA performed on colonies gave 73 positive results, giving a sensitivity of 90.1% and a specificity of 100%. By comparison, the sensitivity and specificity of cytotoxigenic culture, stool cytotoxin and stool toxin A were respectively 96.2% and 100%, 61.7% and 100%, and 66.7% and 95.9%.

**Conclusions:** Performing EIA on colonies recovered after 24 h culture allows us to improve the detection of toxigenic strains in patients suspected of having *C. difficile*-associated disease.

**Key words:** *Clostridium difficile*, toxin A, cytotoxin, enzyme immunoassay

### **INTRODUCTION**

*Clostridium difficile* is the main etiologic agent of antibiotic-associated colitis or diarrhea and is considered as the most frequent agent of infectious diarrhea occurring in hospitalized patients [1]. The pathology is due to the production of at least two toxins: toxin A is an enterotoxin which induces intestinal tissue damage and a fluid response, and toxin B is a cytotoxin which lacks any enterotoxic activity but is believed to exert an additive effect in vivo [2].

Common laboratory strategies for diagnosing *C. difficile*-associated disease (CDAD) include stool culture and fecal toxin detection [3]. Culture is performed by plating fecal specimens on selective media, and a 48-h incubation is recommended. It has the best sensitivity but lacks specificity due to the possible carriage of non-toxigenic isolates [1]. Subsequent determination of the toxigenic status of an isolate may be performed but entails a delay of several days. Cytotoxin is detected by the cytopathic effect of a fecal filtrate on most cultured mammalian cells [4]. Finally, several enzyme immunoassays (EIAs) allow the detection of fecal toxin A or toxin B [5-9]. In the present study, we have introduced modifications to the classical procedures with the aim of improving the detection of toxigenic strains in patients suspected of having CDAD. Firstly, we have used selective media, including sodium taurocholate, which had been shown previously to promote clostridial spore germination [10,11], and reduced the incubation time to 24 h instead of 48 h by using the Mart<sup>®</sup> Anoxomat

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system (Mart, Lichtenvoorde, Netherlands), which allows us to obtain an anaerobic atmosphere in jars within minutes. Secondly, we have tested strain toxigenicity on every positive culture by performing a toxin A EIA directly on colonies. We have compared the performance of this 24-h culture method combined with EIA on colonies for the detection of toxigenic strains in patients suspected of having CDAD with three other methods: standard toxigenic culture, direct stool cytotoxin detection and direct stool toxin A detection.

## MATERIALS AND METHODS

### Reference strains

Ten *C. difficile* reference strains from the ATCC (nos 43593, 43594 and 43596 to 43603), corresponding to ten serogroups, were used to assess the ability of the EIA to detect toxin A production by colonies. Half of these strains were cytotoxin producers.

### Patients and stool specimens

Consecutive fresh diarrheal stool specimens obtained in our routine laboratory from hospitalized patients were investigated over a 7-month period. The stool specimens were stored at 4°C and processed within 24 h after collection. All patients were over 4 years of age. Most of them were hospitalized in oncology, hematology or intensive care units, where their stools were weekly systematically investigated. Clinical data of patients with positive results were reviewed. Antibiotic-associated diarrhea (AAD) was diagnosed when antibiotic had been given in the past 8 weeks and when patients had presented at least six stools in the prior 48 h.

### Culture

Stool specimens were spread out on two plates: a fructose egg yolk agar medium described by George et al. [12] which had been modified to include the selective agents cycloserine at 400 µg/mL and cefotaxime at 3.6 µg/mL (CCFA), and a second plate similar to the previous one except that it included, in addition, 1 mg/mL sodium taurocholate (TCCFA) [10,11]. CCFA plates were incubated anaerobically at 37°C for 48 h and TCCFA plates at 37°C for only 24 h. Both plates were incubated in jars where the anaerobic atmosphere was obtained by using the Mart<sup>®</sup> Anoxomat, which is a recently available device allowing us to obtain and control anaerobic conditions in jars within about 2 min. The system is based on air aspiration by a pump, automatic control of airtightness and catalyst activity and injection of a gas mixture (10% CO<sub>2</sub>, 10% H<sub>2</sub>, 80% N<sub>2</sub>). Colonies of *C. difficile* were identified by morphology examination and by fermentation-product analysis using gas-liquid chromatography [13].

### Cytotoxin assay

Stool cytotoxin assay was performed by testing fecal filtrate for cytotoxin on a confluent monolayer of HeLa cells [13]. The final dilution of the stools was 1/100. The cells were examined after one night and after 48 h of incubation. A positive assay was confirmed by neutralization with *C. sordellii*-specific antiserum (Wellcome, Belgium). In vitro cytotoxin production of the *C. difficile* isolates recovered from CCFA medium was tested after 5 days' growth of the strains in prereduced brain-heart infusion broth (BHI) (Difco, USA). The culture supernatant was assayed in the same way as fecal filtrate.

### Toxin A assay

Stool toxin A was detected with the Premier<sup>™</sup> *C. difficile* Toxin A EIA (Meridian Diagnostics, Cincinnati), in less than 3 h, according to the instructions of the manufacturer. Results were analyzed by spectrophotometric reading at the wavelengths of 450/630 nm. A positive result was recorded when absorbance was  $\geq 0.100$ , a negative result when it was  $<0.070$ , and an undetermined result when it was between 0.070 and 0.100. Positive and negative controls were included in each series.

To perform the toxin A EIA on colonies, one colony, 2 to 3 mm in diameter, or three smaller colonies recovered from 24-h TCCFA medium, were picked up as soon as the anaerobic jar was opened and mixed with 200 µL of sample diluent. Toxin A was then detected in this suspension using Premier<sup>™</sup> *C. difficile* Toxin A EIA in the same way as stool toxin A.

### Serogrouping

The serogroup of each strain was determined according to the method described by Delmée et al. and confirmed by PAGE as reported previously [14].

## RESULTS

To assess the ability of the EIA to detect toxin A production by colonies, the 10 reference strains were grown on CCFA and colonies were tested with Premier™ Toxin A EIA. The four strains, of serogroups A, C, G and H, all cytotoxin producers, yielded a positive result, with ODs ranging from 2.570 to >3, the mean OD being 2.817. The six other strains displayed clear negative results, with ODs always less than or equal to 0.010. Five of these six negative strains, of serogroups B, D, I, K and X, did not produce cytotoxin in vitro, whereas the strain of serogroup F did. Indeed, strains of serogroup F have been previously reported to be cytotoxin but not toxin A producers [15].

**Table 1** Discordant results between the two toxigenic culture methods

Number of Samples	CCFA culture	In vitro cytotoxin detection	TCCFA culture	Colony toxin A EIA	Sources of failure
3	-		+	+	False-negative CCFA culture
2	+	+	-	-	False-negative TCCFA culture
5	+	+	+	-	False-negative colony EIA
1	+	+	+	-	Strain of serogroup F producing cytotoxin but not toxin A

Five hundred and forty-eight stool samples obtained from 411 patients were included in the study. *C. difficile* was isolated from 106 fecal specimens on 24-h TCCFA. In all cases, there were enough colonies to test the toxin A production of the isolates. The EIA was positive for 73 isolates, with ODs ranging from 0.192 to >3, the average being 2.310. Thirty-three colonies displayed clear negative results, with all ODs inferior to 0.015. Thus, culture EIA, performed within 24 h, yielded 73/548 (13.3%) positive results.

In comparison, 105 isolates were recovered from 48-h CCFA. Each isolate was tested for in vitro cytotoxin production after 5 days' incubation in BHI. Seventy-eight were found to be cytotoxin producers. Thus, the standard toxigenic culture yielded 78/548 (14.2%) positive results, within 7 days.

Direct fecal cytotoxin detection yielded 50/548 (9.1%) positive results, within 24 h. Direct fecal toxin A detection, performed in less than 3 h, yielded 73/548 (13.3%) positive results.

The two toxigenic culture methods, TCCFA followed by colony toxin A EIA and CCFA followed by in vitro cytotoxin detection, gave concordant results for 537 stool specimens, 70 positive and 467 negative. For the 11 discordant stool specimens, repeating both methods allowed us to resolve the discrepancies (Table 1). For five discordant specimens, discrepant results were due to false-negative cultures, two on TCCFA and three on CCFA. The five isolates recovered after duplicate culture were all cytotoxin and toxin A producers. For five other discordant specimens, both cultures were positive but discrepant results were due to false-negative toxin A detection on colonies: indeed, toxin A detection was positive when repeating the test. The last discrepant result was due to the presence of a serogroup F isolate which produces cytotoxin but not toxin A [15].

Hence, a total of 108 stools gave a positive culture by at least one of the culture methods, and 81 isolates were shown to produce cytotoxin in vitro. These final results have been considered as the reference.

On that basis, the performances of the four methods, at the first run, have been compared (Table 2). The culture followed by EIA reached a sensitivity of 90.1% and a specificity of 100%. The standard toxigenic culture reached a sensitivity of 96.2% and a specificity of 100%. The fecal cytotoxin detection gave a sensitivity of 61.7% and a specificity of 100%. From the 31 false-negative results, two were in fact non-specific results. The fecal toxin A detection showed a higher sensitivity, 66.7%, but a lower specificity, 95.9%. Indeed, there were 27 false-negative and 19 false-positive results. Of the 12 (2.1%) undetermined results, which were considered as negative, only one was a false-negative.

The prevalence of the toxigenic *C. difficile* carrier state was 13%. Correlations between detection of cytotoxin, toxin A and toxigenic strain in stool and clinical data have been established for 19 patients from whom 29 positive stool specimens had been collected. The results showed that fecal cytotoxin detection and fecal toxin A detection correlated in 25/29 stool specimens, 8 both positive, 17 both negative, although toxigenic culture was positive for the 29 specimens (Table 3). Whatever the results of direct fecal cytotoxin and/or fecal toxin A detection might be, the clinical chart of each patient was compatible with CDAD. Furthermore, for four patients, two discrepant stool specimens were collected within a few days' interval, both being positive for toxigenic culture, but only one being positive for direct fecal toxin detection.

**Table 2** Comparison of the performances of the two toxigenic culture methods and the two fecal toxin detection methods

	Total Reference <sup>a</sup>	TCCFA culture and colony toxin A EIA		CCFA culture and in vitro cytotoxin detection		Fecal cytotoxin detection		Fecal toxin A detection	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
		548							
Positive	81	73	8	78	3	50	31	54	27
Negative	467	0	467	0	467	0	467	19	448
Sensitivity			90.1%		96.2%		61.7%		66.7%
Specificity				100%		100%		100%	95.9%
Predictive positive value			100%		100%		100%		74%
Predictive negative value			98.3%		99.3%		93.8%		94.3%

<sup>a</sup>Final toxigenic culture results.

**Table 3** Correlation between fecal cytotoxin detection and fecal toxin A EIA for 29 stool specimens with positive toxigenic culture

Number of samples	Fecal cytotoxin detection	Fecal toxin A EIA
8	+	+
17	-	-
3	+	-
1	-	+

## DISCUSSION

In the present study, we have evaluated the combination of two original approaches for the detection of toxigenic strains in patients suspected of having CDAD: a shorter fecal culture on selective media supplemented with sodium taurocholate and incubated using a new anaerobic device, combined with the determination of the toxin A status of the isolates by performing the Premier™ Toxin A EIA directly on colonies. As shown by our results, the performance of this combined method, which gives the result within 24 h, is excellent.

The use of the **Mart® Anoxomat** system allows us to obtain anaerobic conditions in jars within minutes. This system has been in routine use in our laboratory for several months, instead of the classical anaerobic GasPak (Becton-Dickinson, Maryland), and **has significantly improved our anaerobe recovery** (unpublished results).

Here, we have combined its use with the addition of sodium taurocholate in the CCFA medium, which has been shown to improve the spore germination [10,11]. As compared with the classical 48-h CCFA culture, we obtained a similar sensitivity, since, of a total of 108 positive cultures, 106 were recovered on 24-h TCCFA versus 105 on 48-h CCFA. Moreover, the colony sizes on the 24-h TCCFA were always sufficient to confirm the identification and to perform the EIA test on colony. It is difficult to draw conclusions on the respective roles of sodium taurocholate and of the use of the **Mart® Anoxomat system** in our results, and this warrants further investigation.

The Premier™ Toxin A EIA is marketed for toxin A detection in feces. Here we have used it directly on colonies grown on selective media, in order to reduce the delay and with the hope of improving the sensitivity. We conclude that the test works well, distinguishing toxigenic and non-toxigenic isolates with excellent sensitivity and specificity. Indeed, only five toxin A-producing strains were not detected by the EIA on colony. The test became positive when repeated, which indicates either a true lack of sensitivity or the fact that the stools included a mixture of toxigenic and non-toxigenic isolates. This suggests that EIA should be performed by testing more than one colony. On the other hand, a sixth colony which belonged to serogroup F may be considered as correctly evaluated by the EIA on colony, since this serogroup was shown to produce only toxin B and to be non-pathogenic. Furthermore, we did not notice any interference when *C. difficile* colonies were not properly isolated.

Being performed within 2 to 3 h, the EIA on colonies can be completed the day after the fecal specimen is received. Although it entails a 24 h delay by comparison with the EIA on stools, it considerably reduces the delay when compared to in vitro cytotoxin detection after several days of subculture and it does not last longer than the fecal cytotoxin detection. Together with a higher sensitivity than EIA on stools, it is worth pointing out that it also

allows an important cost reduction, since only those specimens which are positive on culture are tested. In this study, only 106 EIAs were performed on 548 stool specimens: this might give an 80% reduction in the EIA expenses.

Direct detection of toxin A in stools has the obvious advantage of allowing a rapid diagnosis, within 2 1/2 h, and remains a first choice in the most severe clinical cases. The present results, however, indicate a significant increase of sensitivity of the EIA on colonies in comparison with the fecal detection of cytotoxin as well as of toxin A. One may object that detection of a strain that produces toxin *in vitro* does not automatically imply that toxin is actually produced in the intestinal tract: this could reflect in some cases the detection of a carrier state instead of a true pathologic case. This point remains a subject of controversy. Nevertheless, we believe that, confronted with any positive results concerning *C. difficile*, whatever the technique, the final interpretation remains to be made by the clinician, who has to include all relevant clinical data. In any case, knowledge of the carriage of a toxigenic isolate by a patient is of interest for the clinician, primarily from an epidemiologic viewpoint, especially in a hospital setting [16]. To some extent, even the identification of carriers of non-toxigenic isolates is worth knowing, since such carriage has been shown to protect against subsequent colonization by a toxigenic strain [17].

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