JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1993, p. 2526–2528
0095-1137/93/092526-03$02.00/0

Vol. 31, No. 9

Use of an Enzyme-Linked Immunoassay for *Clostridium difficile* Serogrouping

M. DELMÉE,[1]* C. DEPITRE,[2] G. CORTHIER,[2] A. AHOYO,[1] AND V. AVESANI[1]

Microbiology Unit, University of Louvain, Avenue Hippocrate 54.90, B-1200 Brussels, Belgium,[1] and Unité d'Ecologie et de Physiologie du Système Digestif, Centre de Recherche de Jouy, 78352 Jouy-en-Josas, France[2]

Received 1 April 1993/Returned for modification 11 May 1993/Accepted 21 June 1993

An enzyme-linked immunoassay (ELISA) with 11 *Clostridium difficile* serogroup-specific antisera was applied for serogrouping of *C. difficile* colonies from 314 consecutive positive fecal samples. Two hundred forty-nine strains (79%) were correctly serogrouped, 57 (18%) belonged to serogroups other than the 11 which were evaluated and gave a negative reaction with all antisera, and 8 isolates (2.5%) did not react with their corresponding antisera. ELISA is a rapid and reliable method for serogrouping *C. difficile* and should allow for the automation of this procedure.

Serogrouping of *Clostridium difficile* on the basis of slide agglutination with rabbit antisera allows the differentiation of 10 serogroups, designated by letters (4, 5). A correlation was demonstrated between serogroups and profiles in polyacrylamide gel electrophoresis (PAGE), another system used for typing *C. difficile* (5). Specific profiles were associated with each of the 10 serogroups except serogroup A, for which 12 different profiles, designated A1 to A12, were observed. Later we showed that cross agglutinations observed among strains of serogroup A were due to a common flagellar antigen and that the shearing of flagella allowed specific slide agglutination of the 12 subgroups of serogroup A with 12 corresponding antisera (1). A specific somatic proteic antigen was identified for each serogroup, and the corresponding band was located on PAGE profiles (2).

In this work we have tried to evaluate the potential to carry out direct typing of *C. difficile* colonies with new antisera raised against serogroup-specific antigens in an enzyme-linked immunoassay (ELISA). This approach may allow rapid typing on colonies and may introduce automation while avoiding cross-reactions due to flagellar antigens.

A total of 141 strains were chosen from our collection and were used in this work for the evaluation of the different parameters of the ELISA. They included the reference strains of the 10 serogroups (A1, B, C, D, F, G, H, I, K, and X) and of the 12 subgroups within serogroup A (A2 to A12). The remaining 120 strains were fecal isolates from various sources distributed among all serogroups. Strains from clusters were avoided. The distribution of the strains is shown in Table 1.

Eleven antisera against specific antigens of serogroups A1, A5, A8, A9, A10, C, D, F, G, H, and K were raised in rabbits, as previously described (2). These were the 11 most frequent serogroups in our *C. difficile* collection.

The procedure used was an indirect solid-phase ELISA. Bacterial suspensions were made in carbonate buffer at pH 9.6 and adjusted by reference to the McFarland scale. Wells of microtiter plates (Immulon M 129 A; Dynatech) were coated with 100 μl of the suspension for 2.5 h at 37°C. The plates were then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tw). One hundred microliters of the antiserum diluted in PBS-Tw was added for 1.5 h at 37°C. After washing with PBS-Tw, 100 μl of antirabbit peroxidase conjugate diluted 1:1,000 in PBS-Tw was then added for 1 h at 37°C. After a final washing, a solution of 3,3′,5,5′-tetramethylbenzidine was mixed with H_2O_2 and added to the wells. The reaction was stopped after a 10-min incubation by adding 2 M H_2SO_4. Optical densities (ODs) were read spectrophotometrically at 450 nm.

Fecal specimens were received in the routine laboratory of our university hospital for diagnosis of *C. difficile* infections. They were cultured on selective media (CCFA) for *C. difficile* at 37°C for 48 h in an anaerobic atmosphere (6). The plates were then examined for the presence of colonies with a characteristic morphology (i.e., yellowish, irregular edges, ground-glass appearance). Identification of *C. difficile* was confirmed by gas chromatography of volatile fatty acids. *C. difficile* isolates were serogrouped by slide agglutination with 10 antisera directed against whole cells, as described previously (4). One additional antiserum corresponding to serogroup S1 as defined by Toma et al. was also used (13). Serogroup A strains were further differentiated on the basis of PAGE (5).

Reference strains corresponding to the 11 available antisera were used to determine adequate antigen and antibody concentrations. Bacterial suspensions corresponding to grades 1, 2, and 3 on the McFarland scale were first compared with each of the antisera diluted 1:1,000. Each test was performed in duplicate. The highest ODs were obtained with bacterial suspensions at McFarland grade 3. The ODs ranged from 0.9 to 1.6 for suspensions at McFarland grade 1 and from 1.9 to >3 at McFarland grade 3. The reproducibility was very satisfactory. Reactions of reference strains with heterologous antisera gave low ODs ranging from 0.01 to 0.21 regardless of the McFarland grade used, with the exception of two cross-reactions when testing the serogroup F reference strain with antiserum C and the serogroup C reference strain with antiserum F: the ODs for the serogroup C strain against antiserum F at McFarland grades 1, 2, and 3 were 0.15, 0.42, and 0.64, respectively, while the ODs for the serogroup F strain against antiserum C were 0.85, 1.2, and 1.5, respectively. McFarland grade 3 suspensions of each of the 21 reference strains indicated in Table 1 were tested against the 11 antisera at four different dilutions: 1:1,000, 1:2,000, 1:4,000, and 1:8,000. The ODs decreased with

* Corresponding author.

TABLE 1. Distribution of 141 *C. difficile* strains used for determining assay parameters

Serogroup	Reference strain	Total no. of strains, including reference
A1	1194 (ATCC 43594)	11
B	1351 (ATCC 43593)	3
C	545 (ATCC 43596)	11
D	3232 (ATCC 43597)	11
F	1470 (ATCC 43598)	11
G	2022 (ATCC 43599)	11
H	2149 (ATCC 43600)	11
I	7322 (ATCC 43601)	3
K	4811 (ATCC 43602)	11
X	5036 (ATCC 43603)	3
A2	8643	2
A3	8676	1
A4	6516	2
A5	7701	11
A6	6517	3
A7	7773	3
A8	8737	11
A9	8785	6
A10	T005	11
A11	SE005	2
A12	8480	3

higher dilutions of antisera, with some variations. Cross-reactions between the serogroup C and F strains and antisera were observed: at the four dilutions from 1:1,000 to 1:8,000, the ODs which were recorded when the serogroup F strain reacted with antiserum C were 1.5, 1.2, 1.0 and 0.69, respectively, while those for the serogroup C strain reacting with antiserum F were 0.55, 0.44, 0.43, and 0.21. The following dilutions were chosen for the final protocol: 1:1,000 for antiserum A10; 1:2,000 for antisera A1, A5, A9, G, and H; and 1:4,000 for antisera A8, C, D, F, and K.

In order to establish cutoff points, a grade 3 McFarland suspension of all of the 141 strains of known serogroup listed in Table 1 was tested against the 11 antisera at the dilutions indicated. The ODs were recorded, and the average, standard deviation, and lower and upper limits of the confidence intervals at 99% were calculated. Serogroups that showed cross-reactions in the preliminary test, e.g., serogroup F strains against antiserum C and serogroup C strains against antiserum F, were excluded from our calculations and analyzed separately. This analysis demonstrated no overlaps between confidence intervals at 99% of positive and negative values. Consequently, the lower limits of the confidence intervals of positive values were chosen as the cutoff points. They are indicated in Table 2. The 11 strains belonging to serogroup C that cross-reacted with antiserum F gave a range of ODs from 0.189 to 0.355, while the 11 serogroup F strains gave ODs ranging from 0.215 to 0.692 with antiserum C. These values were below the cutoff points but were markedly higher than the usual values obtained for negative reactions.

Direct typing of *C. difficile* colonies isolated from 314 consecutive positive fecal samples was performed. The results were compared with those of slide agglutination and PAGE analysis. Samples were obtained over a 4-month period, and ELISA was performed with colonies following incubation in anaerobic jars. Usually two or three colonies were picked up to obtain a satisfactory suspension at McFarland grade 3. In 22 cases, it was not possible to obtain a bacterial suspension heavier than grade 2 on the McFarland scale but ELISA was, nevertheless, performed.

Table 2 shows the distribution of the 314 *C. difficile* isolates among serogroups as determined by slide agglutination and PAGE and gives the number of strains that gave a positive reaction with regard to each of the antisera. Two hundred fifty-seven strains (82%) belonged to 1 of the 11 serogroups for which an antiserum was available. The most prevalent serogroups were D (63 isolates), G (58), H (50), C (21), K (16), A1 (16), and A10 (15). Twelve strains belonged

TABLE 2. Correlation between serogrouping by slide agglutination and PAGE and by ELISA for 314 consecutive isolates of *C. difficile* from fecal specimens

Serogroup	Total no.	No. of strains which gave a positive result with antiserum[a]										Range of positive ODs	Cutoff point
		A1	A5	A8	A10	C	D	F	G	H	K		
A1	16	15										0.4–2.5	0.22
B	3												
C	21					21		3				1.3–2.9	0.83
D	63						62					0.8–3	0.78
F	3							3				1.5–2	0.68
G	58								58			0.5–1.4	0.50
H	50									49		0.6–2.8	0.55
I	3												
K	16										15	0.6–2.0	0.51
X	8												
A2	5												
A3	1												
A5	6		5									1.4–1.89	0.87
A6	2												
A7	1												
A8	9			9								0.7–1.8	0.64
A10	15				12							0.6–1.1	0.42
A11	2												
A12	5												
S1	15												
Unknown	12												

[a] Antiserum A9 was also tested, but no strains gave positive results.

to as-yet-unknown serogroups. From those 257 isolates, 249 (97%) were correctly serogrouped by ELISA. This number represents 79% of all isolates. Eight isolates (2.5%) belonging to serogroups A10 (three isolates), A1, D, H, K, and A5 gave negative values for all antisera. ELISAs were repeated after reisolation of strains on nonselective media and strains were then correctly classified.

Cross-reactions were observed for strains of serogroups C and F. Three of the 21 serogroup C strains cross-reacted with antiserum F, but the ODs were only slightly higher than the cutoff point of antiserum F. This was in contrast with high positive values obtained with antiserum C, and the strains were correctly serogrouped. None of the three serogroup F strains had ODs higher than the cutoff point of antiserum C, although these values were higher than those usually obtained for negative reactions.

These results indicate that this ELISA system offers some advantages compared with other typing techniques. The procedure is rapid and can be performed directly with colonies from primary fecal cultures, and a typing result can be obtained within 6 to 7 h. In comparison, slide agglutination requires reisolation and subculturing that delay a result for approximately 4 days (4). PAGE, immunoblots, and restriction endonuclease analysis also require several days before a result is obtained (8, 11, 12). In addition, ELISA is easier to perform than the other techniques since it is routinely used in most laboratories, and it can be automated to some extent.

The greatest advantage is probably the fact that cross agglutinations which are due to flagellar antigens that obscure results from the slide agglutination procedure (1) are totally suppressed by ELISA. Sixty-two of the 314 strains (20%) that we studied belonged to one of the subgroups of serogroup A. Of those, 46 belonged to serogroups for which antisera were available (A1, A5, A8, A9, or A10), 41 of which were correctly serogrouped as indicated in Table 2. Subdifferentiation of strains among serogroups on the basis of PAGE profiles was reported by several authors (7, 10, 11) and is mainly due to common flagellar antigens masking reactions with somatic antigenic determinants which are observed on PAGE profiles or by immunoblots (1). Our results confirm the excellent correlation between serogroup and PAGE profiles and encourage the effort to determine a comprehensive scheme for typing *C. difficile*.

The cross-reactions that were observed between serogroups C and F are not surprising since such crossing has already been observed on immunoblots in a previous study (2). This difficulty is easily circumvented by the fact that the reaction with the heterologous antiserum is much lower compared with that with the homologous one and, most often, is below the cutoff point. Absorption of antisera or use of monoclonal antibodies might be helpful to suppress these cross-reactions.

We were limited by the availability of only 11 antisera. This was mainly due to the amount of work necessitated by the preparation of antisera. We limited our choice to the 11 most frequent serogroups in our routine practice. Our results confirm that they cover 82% of our isolates. Obviously, more antisera have to be prepared and evaluated to cover a larger part of the isolates.

From a medical viewpoint, the main interest in rapid typing of colonies in routine practice is certainly the ability to identify patients who have been contaminated during hospital outbreaks. Nosocomial transmission of *C. difficile* has been well demonstrated as the main mode of contamination by this pathogen (9). Severe outbreaks have been reported in which a single epidemic strain was implicated, and some serogroups are thought to be more epidemic. For example, toxigenic strains of serogroup C, which are more resistant to antibiotics than other serogroups, were more often implicated in outbreaks (3). The usual methods for typing, such as immunoblotting, PAGE, and serogrouping by slide agglutination, entail too long a delay for obtaining a result and, hence, cannot be used for the direct identification of clusters, in contrast to ELISA. Early identification of patients contaminated by an epidemic strain, especially when an index case has been diagnosed within a ward, should allow effective preventive measures.

REFERENCES

1. **Delmée, M., V. Avesani, N. Delferriere, and G. Burtonboy.** 1990. Characterization of flagella of *Clostridium difficile* and their role in serogrouping reactions. J. Clin. Microbiol. **28**:2210–2214.
2. **Delmée, M., V. Avesani, I. Ernest, and M. Surleraux.** 1990. Detection of specific antigens for ten serogroups of *Clostridium difficile*. Mol. Cell. Probes **4**:1–10.
3. **Delmée, M., G. Bulliard, and G. Simon.** 1986. Application of a serogrouping technique for *Clostridium difficile* in an outbreak of antibiotic associated diarrhoea. J. Infect. **13**:5–10.
4. **Delmée, M., M. Homel, and G. Wauters.** 1985. Serogrouping of *Clostridium difficile* strains by slide agglutination. J. Clin. Microbiol. **21**:323–327.
5. **Delmée, M., Y. Laroche, V. Avesani, and G. Cornélis.** 1986. Comparison of serogrouping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. J. Clin. Microbiol. **24**:991–994.
6. **George, W. L., V. L. Sutter, D. Citron, and S. M. Finegold.** 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. **9**:214–219.
7. **Kato, H., J. J. Cavallaro, N. Kato, S. L. Bartley, G. E. Killgore, K. Watanabe, and K. Ueno.** 1993. Typing of *Clostridium difficile* by Western immunoblotting with 10 different antisera. J. Clin. Microbiol. **31**:413–415.
8. **Kuijper, E. J., J. H. Oudbier, W. N. Stuifbergen, A. Jansz, and H. C. Zanen.** 1987. Application of whole-cell DNA restriction endonuclease profiles to the epidemiology of *Clostridium difficile*-induced diarrhea. J. Clin. Microbiol. **25**:751–753.
9. **McFarland, L. V., M. E. Mulligan, R. Y. Y. Kwok, and W. E. Stamm.** 1989. Nosocomial acquisition of *Clostridium difficile* infection. N. Engl. J. Med. **320**:204–210.
10. **Mulligan, M. E., S. Halebian, R. Y. Y. Kwok, W. C. Cheng, S. M. Finegold, C. R. Anselmo, D. N. Gerding, and L. R. Peterson.** 1986. Bacterial agglutination and polyacrylamide gel electrophoresis. J. Infect. Dis. **153**:267–271.
11. **Mulligan, M. E., L. R. Peterson, R. Y. Y. Kwok, C. R. Clabots, and D. N. Gerding.** 1988. Immunoblots and plasmid fingerprints compared with serotyping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. J. Clin. Microbiol. **26**:41–46.
12. **Tabaqchali, S., D. Holland, S. O'Farrell, and R. Silman.** 1984. Typing scheme for *Clostridium difficile*: its application in clinical and epidemiological studies. Lancet **i**:935–938.
13. **Toma, S., G. Lesiak, M. Magus, H.-L. Lo, and M. Delmée.** 1988. Serotyping of *Clostridium difficile*. J. Clin. Microbiol. **26**:426–428.