Serogrouping of *Clostridium difficile* Strains by Slide Agglutination

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Six different agglutinating antisera were obtained by immunizing rabbits with Formol-treated strains of *Clostridium difficile*. After appropriate absorption, these antisera were used to define six serogroups designated by the letters A, B, C, D, F, and G. Altogether, 315 strains of *C. difficile* from various origins were tested for slide agglutination by these antisera; 312 (99%) of them were agglutinated by one of these antisera. A and C were the most common serogroups. An excellent correlation, ranging from 85 to 100%, was found between the serogroup and the toxigenicity of the strains. The correlation between serogroup and sorbitol fermentation was higher, ranging from 89 to 100%. The results of this typing were compared with the clinical origin of the strains. Only strains of serogroups A, C, and D were isolated in 153 cases of antibiotic-associated diarrhea. This series included strains from three outbreaks; all the strains in two of the outbreaks belonged to serogroup C, and in the third, all the strains belonged to serogroup A. Strains of serogroups B, F, and G were only found in the stools of asymptomatic neonates or young children. In the latter samples, strains of serogroups A and D were found in the same ratio as in adults with antibiotic-associated diarrhea, but strains of serogroup C were seldom isolated. In patients treated with antineoplastic drugs and suffering from diarrhea, the distribution of the strains was the same as in cases of antibiotic-associated diarrhea.

Clostridium difficile is known to be the primary cause of pseudomembranous colitis and is often isolated from patients suffering from colitis associated with antimicrobial or cancer chemotherapy (2, 4, 23, 24, 40). According to several authors, the carriage rate in adults ranges from 0 to 11% (9, 26). Clusters of cases or large outbreaks in hospitals have been previously described, and nosocomial spread has been proposed as a mode of transmission (5, 6, 15, 16, 25, 28, 31, 32). The role of this microorganism in relapse of inflammatory bowel disease is less evident (10, 14, 35, 41).

In neonates and young children, however, there is some controversy over the pathogenic role of *C. difficile*. Asymptomatic carriage is very high (50 to 75% in neonates) (16-18, 30). Some cases of pseudomembranous colitis have been previously described (21). Toxin of *C. difficile* has been found in the stools of children or neonates with chronic diarrhea, peritonitis, Hirshprung disease, or necrotizing enterocolitis, but the exact role of the toxin producing *C. difficile* is controversial (1, 3, 7, 12, 17, 20, 34, 36, 39).

To elucidate those problems and to acquire a better understanding of the epidemiology, different typing schemes for *C. difficile* have been proposed. Wüst et al. (42) investigated an outbreak of antibiotic-associated diarrhea (AAD), with various existing methods used in the typing of other bacterial species. Sell et al. (33) used the phage sensitivity pattern to differentiate strains, and more recently, Tabaqchali et al. (37) used electrophoresis of labeled cellular proteins to differentiate nine groups of *C. difficile*. Nakamura et al. (27) demonstrated that Formol-treated strains of *C. difficile* could be agglutinated with rabbit antisera. We have pursued this approach by using a simple slide agglutination technique and applying it to the study of strains from various origins.

MATERIALS AND METHODS

Bacterial isolates. A total of 311 *C. difficile* strains isolated from clinical material were tested. Three reference strains

were obtained from the American Type Culture Collection (ATCC 9689, ATCC 17857, and ATCC 17858), and one was obtained from the National Collection of Type Cultures, Colindale, United Kingdom (NCTC 11223). The first group of 153 strains was isolated from cases of AAD in our laboratory or received from 23 other laboratories in Belgium. This series included strains from three outbreaks of AAD from three different hospitals (5 cases in each of the first two and 43 cases in the third). A second group of 96 strains was isolated from feces of neonates who were involved in a carriage surveillance program regardless of enteric symptoms. Two further groups included 36 strains isolated from young children (less than 3 years old) with diarrhea and 26 strains from leukemic patients receiving a cancer chemotherapy.

The strains were isolated from stool on a selective medium described by George et al. (8). They were each identified by biochemical characteristics and fermentation product analysis by gas-liquid chromatography (4, 11).

Bacterial antigen preparation. Each strain was inoculated into the medium described by Nakamura et al. (27), containing 3% (wt/vol) proteose peptone no. 2 (Difco Laboratories), 0.5% (wt/vol) yeast extract (Difco), 1% (wt/vol) glucose, 0.5% (wt/vol) NaCl, and 0.05% L-cystein · HCl (pH 7.2), and was incubated overnight aerobically at 36°C. A volume of 0.05 ml of this culture was inoculated into 10 ml of the same medium and incubated at 36°C for a further 6 h. Bacteria were washed twice with saline and suspended in 0.4% Formol-saline to obtain an opacity corresponding to the no. 7 of the McFarland scale.

Rabbit immunization. Rabbits were injected intravenously with 0.5, 1, 2, and 2 ml of the Formol-treated suspension, given at 4-day intervals. The rabbits were bled 10 days after the last injection.

Slide agglutination test. Each serum was diluted 1 in 50 in saline, and then serial twofold dilutions were made. One drop of each dilution was mixed on a slide with one drop of the Formol-treated strain, the slide was tilted back and forth, and the agglutination reaction was recorded with the aid of a

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TABLE 1. Results of cross-agglutination between the eight strains used for immunization and the corresponding antisera

Strain no.	Slide agglutination ^a with the following antisera:							
	ATCC 9689	1351	NCTC 11253	1194	0545	3232	1470	2022
ATCC 9689	+++	-	-	+++	-	-	-	++
1351	-	+++	-	-	-	-	+	-
NCTC 11253	-	-	+++	-	+++	-	-	-
1194	+++	-	-	+++	-	-	-	++
0545	-	-	+++	-	+++	-	-	-
3232	-	-	-	-	-	+++	-	+
1470	-	+	-	-	-	-	+++	-
2022	+	-	+	+	+	+	-	+++

^a +++ and ++, complete clumping against a clear background; +, incomplete clumping; -, negative reaction.

convex mirror after 3 min. The agglutination reaction was graded on an increasing scale of - to 3+, with 3+ and 2+ indicating complete clumping of the bacterial cells against a clear background and 1+ indicating partial clumping. A reaction of 2+ or more was considered to be positive. A stock dilution, four times more concentrated than the highest dilution giving a positive result, was used as working dilution.

Agglutinin absorption test. Approximately 1 ml of a dense suspension of the Formol-treated strains prepared as described above was incubated for 24 h at 36°C with 1 ml of the serum diluted 1 in 10. After centrifugation, the serum was tested against both the absorbing and the homologous strains. If necessary, the absorption was repeated.

Cytotoxin production assay. All strains were inoculated in the medium described above and incubated aerobically at 36°C for 48 h. After centrifugation, the supernatant was filtered through a 0.22- μ m filter. Cytotoxicity testing was performed on confluent monolayers of HeLa cells, 0.1 ml of each filtrate being added to 1 ml of Eagle minimal essential medium (GIBCO Laboratories) containing 10% calf serum. The same sample was inoculated on another monolayer with 0.1 ml of *Clostridium sordellii* antitoxin (Wellcome Research Laboratories). The cultures were incubated at 36°C and observed at 6, 24, and 48 h for cytotoxic changes. A negative result was recorded when no morphological changes in the monolayer were detected at 48 h. A positive toxin assay was recorded when morphological changes were observed, prevented by neutralization with specific antiserum.

Sorbitol fermentation test. Three or four colonies were inoculated in a semisolid thioglycolate medium containing 1% sorbitol and incubated at 36°C for 48 h. Four drops of 0.1% phenol red solution was added. A yellow color was considered to be positive.

RESULTS

The toxigenic strains ATCC 9689 and NCTC 11223, a toxigenic and a nontoxigenic strain isolated in stools of neonates (1194 and 1351), and a toxigenic strain isolated from a case of pseudomembranous colitis (0545) were first used to immunize five rabbits. A total of 231 Formol-treated strains were agglutinated by one or more of these antisera. The remaining 84 strains were not agglutinated by any of these antisera. Three of these strains were selected to prepare new antisera: two toxigenic strains (1470 and 2022) and one nontoxigenic (3232). Each serum was titrated with the homologous strain, and a stock dilution was prepared. The eight diluted antisera were assayed against each of the

eight Formol-treated strains; the results of cross-agglutination studies are reported in Table 1.

Sera prepared against strains ATCC 9689 and 1194 appeared to be identical. Serum ATCC 9689 was absorbed with strain 2022 to eliminate the cross-agglutination and was called serum A. Sera NCTC 11223 and 0545 proved to be identical. Serum 0545 was selected and, after absorption with strain 2022, was called serum C. Sera 1351 and 1470 were called B and F, respectively, after cross-absorption with the heterologous strains. Finally, serum 3232, adsorbed by strain 2022, and serum 2022, absorbed by strains 1194 and 3232, were called, respectively, D and G. Each absorbed antiserum was retitrated, and stock dilutions were prepared. Six serogroups called A, B, C, D, F, and G were defined by these absorbed antisera.

For each serogroup, antisera, titers of stock dilutions, and absorbing strains used are summarized in Table 2.

All the strains were examined for agglutination by each of the six antisera, toxin production, and sorbitol fermentation. Strains ATCC 9689, ATCC 17857, and ATCC 17858 were all of serogroup A and were toxin producers and fermented sorbitol. Strain NCTC 11223 belonged to serogroup C and produced toxin and fermented sorbitol. Results obtained for all the other strains studied are reported in Table 3. Most of the strains of serogroups A, C, F, and G were toxigenic, with a correlation ranging from 100% for serogroup G to 85% for serogroup A. All the strains of serogroups B and D were nontoxigenic. It must be emphasized that 12 of 17 nontoxigenic strains of serogroup A and 4 of 5 nontoxigenic strains of serogroup C were isolated from neonates or children. The correlation between serogroup and sorbitol fermentation was better still; 89% of the strains of serogroup A and 95% of the strains of serogroup C fermented sorbitol, but none of the strains of serogroups B, D, F, and G did so. Of the 17 sorbitol-negative strains of serogroups A and C, 8 had been isolated from neonates. Hence, 93% of the toxigenic strains isolated from cases of AAD fermented sorbitol.

In the three outbreaks of AAD investigated, all the strains were toxigenic and belonged to the same serogroup: serogroup A for the first and serogroup C for the other two. In sporadic cases of AAD, only strains of serogroups A, C, and D were found; 77% of the strains were toxigenic.

Strains of serogroups B, F, and G were isolated only from children with diarrhea or from neonates. One strain of serogroup F and one strain of serogroup G was isolated from leukemic patients treated by cancer chemotherapy and suffering from diarrhea; in both cases the patients were young children.

A comparison of the typing results with the clinical origin is summarized in Table 4.

DISCUSSION

Differentiation of *C. difficile* strains by means of a reliable typing scheme provides the opportunity to gain a greater

TABLE 2. Characteristics of antiserum for each serogroup

Serogroup	Antiserum no.	Absorbing strain no.	Titer of stock dilution
A	1194	2202	1/200
B	1351	1470	1/100
C	0545	2022	1/50
D	3232	2022	1/100
F	1470	1351	1/200
G	2022	1194 and 3232	1/200

TABLE 3. Results of serogrouping and relationship among serogroup, toxigenicity, and sorbitol fermentation of *C. difficile* strains.

Serogroup	Total no. of strains (%) ^a	No. of toxigenic strains (%) ^b			No. of nontoxigenic strains ^b		
		Total	Sorbitol ^c		Total	Sorbitol ^c	
			(+)	(-)		(+)	(-)
A	110 (35)	93 (85)	83 (75)	10 (9)	17 (15)	15 (13)	2 (2)
B	14 (5)	0	0	0	14 (100)	0	14 (100)
C	105 (34)	100 (95)	100 (95)	0	5 (5)	0	5 (5)
D	31 (10)	0	0	0	31 (100)	0	31 (100)
F	32 (10)	30 (94)	0	30 (94)	2 (6)	0	2 (6)
G	16 (5)	16 (100)	0	16 (100)	0	0	0
NA ^d	3 (1)	1	1	0	2	2	0

^a Percentage of the total number.^b Percentage of the number of this serogroup.^c (+), Sorbitol-fermenting strains; (-), sorbitol-nonfermenting strains.^d NA, Nonagglutinable strains.

understanding of the mode of transmission, epidemiology, and pathogenicity in children and neonates. Hafiz and Oakley (11) pointed out in 1976 that *C. difficile* could be distinguished on the basis of its antigenic structure, and Nakamura et al. (27) in 1981 obtained three different agglutinating antisera after rabbit immunization with Formol-treated cells. Using the same methodology to raise antisera in rabbits, we performed a simple slide agglutination technique.

Slide agglutination is a simple and rapid method for serogrouping of bacteria. Using this technique, we achieved a suitable typing system for *C. difficile*. The results are easily readable and can be obtained in less than 3 min. Problems of cross-agglutination are minor and are solved by the use of absorbed antisera.

We produced six different antisera which agglutinated 99% of the 315 strains tested. This represents the higher number of serogroups described previously. Nakamura et al. (27) found three different serogroups, but 17% of the strains tested did not agglutinate in any of the antisera. The strains used by Nakamura et al. were different from those used in our study except for strains ATCC 9689 and ATCC 17857. Tabaqchali et al. (37), studying electrophoretic profiles of the labeled cellular proteins, recently obtained nine different groups of *C. difficile*, and it would be interesting to compare these nine groups with our six serogroups.

TABLE 4. Relationship among serogroup, toxigenicity, and the clinical origin of the strains

Clinical origin	No. of strains of the following serogroup and toxigenicity ^a									
	A+	A-	B-	C+	C-	D-	F+	F-	G+	NA ^b
Cases of AAD										
Sporadic cases	33	4	0	40	1	21	0	0	0	1
First outbreak	5	0	0	0	0	0	0	0	0	0
Second outbreak	0	0	0	5	0	0	0	0	0	0
Third outbreak	0	0	0	43	0	0	0	0	0	0
Neonates	29	11	12	2	4	1	22	1	13	1
Children with diarrhea	14	1	2	2	0	6	8	0	2	1
Leukemic patients with diarrhea	12	1	0	8	0	3	0	1	1	0

^a +, Toxigenic strains; -, nontoxigenic strains; letters refer to the serogroups.^b NA, Nonagglutinable strains.

The correlation between serogroup and toxigenicity is obvious. Strains of serogroups B and D are all nontoxigenic. Conversely, there is a high probability that strains of serogroups A, C, F, and G will be toxigenic. Since toxin production has been suggested by several authors (19) to play a part in pathogenicity, serogroups usually associated with toxin production might be of greater concern in the epidemiological involvement of this microorganism.

Sorbitol fermentation is known to be variable in *C. difficile*. Nakamura et al. (27) found some correlation between serogrouping and sorbitol fermentation. Our results confirm this finding. Only strains of serogroups A and C are able to ferment sorbitol, and the toxigenic strains isolated in cases of AAD always belong to one of these two serogroups.

One of the most useful applications of the serogrouping technique would be the study of the epidemiology of this infection. All of the strains isolated from an outbreak belonged to serogroup A, and all the strains isolated from the other two outbreaks belonged to serogroup C. In one of those outbreaks (43 cases), all the cases occurred on the same ward of the hospital, and the strains were of serogroup C. Three other cases of AAD occurred at the same time in other wards of the same hospital; two strains were of serogroup A, and the third was of serogroup C. These findings are in agreement with the results of other authors who suggest the nosocomial spread of the organism (13, 15, 29, 32, 33, 37, 42). From this point of view, serogrouping of environmental strains should be investigated.

Tabaqchali et al. (37) recently found that two groups of *C. difficile* were associated with AAD, whereas four others were isolated mainly from mothers and newborn infants. Our results are in agreement with these findings. Only three serogroups (A, C, and D) were found in 153 cases of AAD, and the pathogenic role of strains belonging to serogroup D, which are all nontoxigenic, is not clear.

The finding that some serogroups of *C. difficile* are isolated only from the feces of neonates or young children should improve our knowledge about the significance of *C. difficile* in infants. Carriage is so high in neonates and young children that it is difficult to assess pathogenicity. For example, Han et al. (12) detected *C. difficile* toxin in 92.3% of necrotizing enterocolitis cases during an outbreak, in comparison with 11.8% in asymptomatic patients, and the outbreak was terminated upon institution of oral vancomycin therapy. Conversely, Mathew et al. (22), Thomas et al. (38), and Sherertz et al. (34) isolated *C. difficile* in the same proportion in asymptomatic and symptomatic patients. However, in a case of pseudomembranous colitis in a

5-week-old infant described by Mandal et al. (21), it is difficult to deny the pathogenic role of the toxigenic strain isolated.

Our results suggest that strains of different serogroups may have different properties. The pathogenicity of strains of serogroups B and D, which are nontoxigenic, should still be assessed. Strains of serogroup C, the most frequent in symptomatic adults, are seldom isolated in neonates and young children. Strains of serogroup A are isolated with the same frequency from adults and children with diarrhea and should be considered as more significant than the other serogroups. Strains of serogroups F and G are both toxigenic and were isolated only in infants. It would be interesting to assess their pathogenicity in animal models.

Finally, the distribution pattern of serogroups in patients treated with cancer chemotherapy seems to be quite similar to that of AAD cases.

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