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Electrophoretic Characterization of *Clostridium difficile* Strains Isolated from Antibiotic-Associated Colitis and Other Conditions

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Clostridium difficile **has been recognized as the cause of antibiotic-associated pseudomembranous colitis and of less severe diarrheal diseases associated with the use of antimicrobial agents. However, healthy carriers of this microorganism have been found, particularly healthy neonates and small children. Various typing systems have been used to clarify the epidemiology of *C. difficile*. We used the electrophoretic patterns of EDTA-extracted proteins to characterize *C. difficile* strains from various sources. Altogether, 110 strains were studied, including 2 reference strains, and 21 different protein profiles were obtained. However, two patterns were the most common: the group 2 pattern, characterized by a major 35-kilodalton polypeptide band, and the group 5 pattern, identified by principal bands of 37 and 56 kilodaltons. The group 2 pattern was characteristic of strains isolated during hospital outbreaks and from sporadic cases of pseudomembranous colitis and antibiotic-associated diarrhea. The group 5 pattern was obtained only from isolates from healthy neonates and children. A correlation between electrophoretic characteristics and virulence can be hypothesized, namely that group 2 strains are more prone to induce diseases and cause outbreaks. It is noteworthy that strains isolated from children with diarrhea of unknown etiology, not related to antibiotic use, belong to the "virulent" group 2; strains from leukemic patients showed a variety of different patterns, and only two belong to group 2. This characterization can be used to aid studies on the virulence and clinical significance of *C. difficile*.**

In the last few years, several studies have shown that *Clostridium difficile* is the etiological agent of antibiotic-associated pseudomembranous colitis (PMC) (1, 17); it has also been made clear that the spectrum of disease caused by this microorganism is very wide, varying from PMC to simple diarrhea with few if any colonic lesions (antibiotic-associated diarrhea [AAD]) (3). Certain host conditions, particularly disruption of the normal bowel flora by antibiotics, are usually regarded as necessary for disease production by *C. difficile* (7).

The role of *C. difficile* in other conditions not related to antibiotics is still under debate, namely in exacerbation of inflammatory bowel diseases (2), in adult chronic diarrhea (11), and in intestinal disturbances of leukemic patients treated with antiblastic drugs (20). Moreover, since most neonates and small children harbor *C. difficile* in their bowels without symptoms (6), it is difficult to evaluate its role in disease conditions such as neonatal necrotizing enterocolitis (25) or infantile acute diarrhea (19).

It is significant that analogous to other bacterial species, some *C. difficile* strains can be more pathogenic than others i.e., more able to produce serious disease, for humans. This cannot be related only to production of toxins, since both adults and children can be healthy carriers of toxigenic strains. A method of distinguishing types within species could be useful in detection of strain differences possibly related to virulence. Since no fully established system is as yet available for typing *C. difficile*, among the variety of systems used by different researchers (serotyping, bacteriophage and bacteriocin typing, patterns of radiolabeled proteins, etc.) (5, 24, 26) we chose electrophoretic patterns of EDTA-extracted surface proteins, a method easy to set up in the laboratory.

In the present paper we report the electrophoretic characterization of *C. difficile* strains isolated during two hospital outbreaks of AAD, from sporadic cases of PMC and AAD, and from individuals with diverse conditions in which the role played by *C. difficile* is still doubtful.

MATERIALS AND METHODS

Sources and strains. *C. difficile* strains were obtained during two hospital outbreaks of AAD (5 and 11 patients) and from sporadic cases of PMC (12 strains) and AAD (14 strains) occurring in different geographic areas of Italy. Diagnosis of PMC was assumed only if confirmed by histology or colonoscopy, or both. Strains from different pathologies and different hosts were also studied: miscellaneous intestinal diseases of adults (3 strains), premature neonates without intestinal abnormalities (13 strains), neonates suspected of having neonatal necrotizing enterocolitis (8 strains), healthy children (7 strains), children with diarrhea not related to antibiotic use (17 strains, isolated from feces or duodenal juices), leukemic patients (13 strains), rabbits with experimental ampicillin-induced diarrhea (4 strains), and one extraintestinal source (sputum). Reference strains NCTC 11223 and VPI 10463 (provided by T. D. Wilkins, Virginia Polytechnic Institute, Blacksburg) were also included. Altogether, 110 strains were studied.

Isolation of *C. difficile*. All *C. difficile* strains were isolated by plating the clinical specimens onto *C. difficile* selective medium containing cefoxitin (16 mg/liter) and cycloserine (500 mg/liter) (Oxoid Italiana S.p.A.) (8) and *p*-hydroxyphenylacetic acid (0.1%) (Sigma Chemical Co., St. Louis, Mo.) (21). *C. difficile* was identified by colony morphology, odor, Gram stain, pattern of volatile fatty acids on gas-liquid chromatography, and biochemical tests (14).

Cytotoxicity test. In vitro cytotoxin production was assayed by growing strains in Robertson cooked meat–0.5% glucose for up to 5 days. Filtered supernatants were tested on HEp-2 cells in microdilution plates (5,000 cells per well in

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TABLE 1. Distribution of strains into groups, according to source

Source	No. of strains	Toxin positive/ toxin negative[a]	No. (%) of strains in:		
			Group 2	Group 5	Other groups
PMC	12	12/0	10 (83)	0	2 (17)
AAD					
first outbreak	5	5/0	5 (100)	0	0
second outbreak	11	10/1	8 (73)	0	3 (27)
sporadic cases	14	12/2	11 (79)	0	3 (21)
Miscellaneous diseases	3	3/0	2 (67)	0	1 (33)
Premature neonates	13	1/12	1 (8)	10 (77)	2 (15)
NEC[b]	8	5/3	3 (37)	3 (37)	2 (25)
Healthy children	7	1/6	1 (14)	5 (71)	1 (14)
Children with diarrhea	17	14/3	11 (65)	1 (6)	5 (29)
Leukemia patients	13	7/6	2 (15)	0	11 (85)
Rabbits	4	4/0	0	0	4 (100)
Sputum	1	1/0	0	0	1 (100)

[a] Number of strains positive/number of strains negative for in vitro cytotoxin production.
[b] NEC, Neonatal necrotizing enterocolitis.

Eagle minimal essential medium containing 1% fetal calf serum) and incubated overnight at 37°C under 5% CO_2. Rounding of at least 50% of cells was considered positive for cytotoxin production (10).

Electrophoresis of proteins. *C. difficile* strains were grown and surface proteins were extracted by the method of Poxton et al. (22). Briefly, strains were grown overnight in 100 ml of proteose peptone-yeast extract broth (13); cells were then harvested by centrifugation at 20,000 × *g* for 8 min, washed twice in phosphate-buffered saline, and extracted with 10 mM EDTA at 45°C for 30 min. Extracts were stored frozen at −20°C until used. Approximately 100 μg of proteins for each sample, as measured by the method of Lowry et al. (18), was run in a sodium dodecyl sulfate–10% polyacrylamide slab gel (160 by 180 by 1.5 mm) in a vertical cell (Bio-Rad Laboratories srl) at constant current until the dye reached the bottom of the gel. Molecular weight standards (Bio-Rad Laboratories) were included in each run. The buffer system described by Laemmli (16) was used. Gels were then stained with Coomassie blue and either dried in a gel drier or stored wet in plastic bags.

RESULTS

The electrophoretic patterns obtained for the strains examined were composed approximately of 20 polypeptide bands, some of which were more evident and therefore were considered for comparison (major bands). The patterns were found to be reproducible in different runs at different times. Comparison among strains in the same gels was easy, but minor variations in different runs required the inclusion in each gel of representative strains with known patterns.

All the strains from the first outbreak and 9 of 12 strains from the second outbreak had identical protein profiles. By extending the study to sporadic cases of PMC and AAD from different geographic areas and to *C. difficile* strains isolated from different hosts and disease conditions, at least 21 different patterns could be recognized, some of them characteristic of a single strain. However, two patterns, called the group 2 and group 5 patterns, were found more frequently and together made up 74% of the strains studied (Table 1). Examples of these two patterns are shown in Fig. 1, together with some unusual profiles.

The group 2 pattern was characterized by a major polypeptide band of approximately 35 kilodaltons (kDa) and was obtained from the majority of strains isolated from individuals with PMC and AAD and during the two outbreaks. Also, 4 of 21 strains isolated from neonates fell within group 2: 1 was from a neonate without intestinal disturbances, and 3 were from neonates suspected of having neonatal necrotizing enterocolitis. Most strains isolated from children with diarrhea, both from feces and from duodenal juices, were found to belong to group 2 (11 of 17).

The group 5 pattern, identified by the presence of two major bands of 37 and 56 kDa (with or without an additional double band of approximately 80 kDa), was characteristic of neonates and infants without intestinal abnormalities. No strain isolated from an adult was found to belong to group 5.

Strains from leukemic patients, regardless of age or intestinal symptoms, presented a variety of different patterns, and only two of these strains had a group 2 profile. Also, strains isolated from rabbits and the only strain from an extra-intestinal source (sputum) showed diverse protein profiles. The two reference strains studied (VPI 10463 and NCTC 11223) were found to have protein profiles consistent with group 2. A clear relationship was found between in vitro toxin production and electrophoretic patterns. All but two strains belonging to group 2 were able to produce toxin; the two nontoxigenic group 2 strains were isolated from children with diarrhea. Strains belonging to group 5 did not produce toxin; the other nontoxigenic strains had different protein patterns, mainly with major polypeptide bands in the range 37 to 49 kDa.

DISCUSSION

In the last few years many researchers have tried to clarify the epidemiology of *C. difficile*-induced disease and have pointed out the need for a typing scheme. Different methods have been suggested, some of which are already used for other microorganisms, such as phage typing or serotyping (5, 24), and some of which are based on new techniques, such as autoradiograms of radiolabeled proteins (26) or restriction endonuclease DNA analysis (15). These methods have proven useful in understanding some aspects of the epidemi-

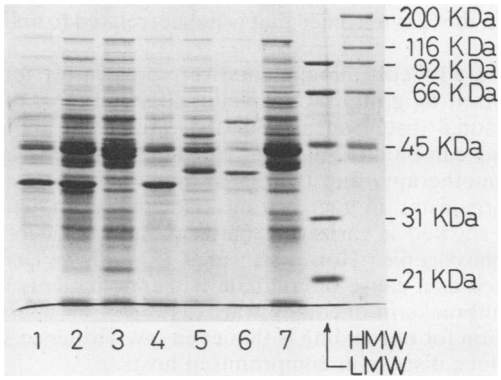

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of *C. difficile* EDTA extracts. Lanes: 1, 2, and 4, group 2 patterns; 6, group 5 pattern; 3, 5, and 7, examples of different patterns. Abbreviations: HMW, high-molecular-weight standards; LMW, low-molecular-weight standards.

ology of *C. difficile*, but no reference scheme has yet been established.

To type our strains we chose a simple and straightforward method, originally described by Poxton et al. (22), i.e., the electrophoretic patterns of EDTA-extracted surface proteins from strains grown under standardized conditions. By this method, a great variety of patterns was obtained from the strains examined. This probably reflects our efforts at studying strains isolated from different patient populations and disease conditions. Despite this heterogeneity, two patterns were detected most commonly: group 2, which is characterized by a major protein of approximately 35 kDa, and group 5, which is identified by two major bands at 37 and 56 kDa. Since protein migration can vary slightly in different electrophoresis experiments and thus slightly alter the molecular weight positions of the major bands, we found it necessary to run a strain with a known pattern in each gel. The inclusion of a reference strain belonging to group 2, such as VPI 10463, will allow different laboratories to establish a similar typing scheme.

In our study, group 2 was found to be associated with strains isolated from individuals with PMC and AAD (both outbreaks and sporadic cases). This suggests a correlation between typing characteristic and virulence, as already pointed out by other researchers (5, 26): some *C. difficile* strains could be more prone to causing severe diseases or spreading in hospitals. Serogroup C of Delmée et al. (4, 5) comprises most of the strains from sporadic cases and from hospital outbreaks of AAD. In our study, strain NCTC 11223, corresponding to serogroup C (5), was found to have a group 2 pattern.

Wexler et al., examining the electrophoretic profiles of whole-cell protein extracts of *C. difficile*, found that the lack of a polypeptide band of approximately 34 kDa was a distinctive feature of nonpathogenic strains, either nontoxigenic or isolated from healthy carriers (27). Despite the differences in method, this finding could be interpreted as indirect evidence that group 2, characterized in our study by the presence of a major 35-kDa protein, is associated with some virulence factors.

Group 2 strains were rarely found in samples from healthy children and premature neonates without intestinal disturbances, but represented the majority of strains isolated from feces or duodenal juices of children with diarrheal diseases of unknown etiology (9, 19). Since the role of *C. difficile* in infantile diarrheas is still being debated, the finding of the "virulent" strain could suggest a pathogenic role for *C. difficile* in these syndromes that is not correlated to antibiotic use.

In our study leukemic patients were only rarely found to be colonized by group 2 *C. difficile*, although most of them had intestinal disturbances. For these patients, abdominal symptoms can be attributed to their illness and the aggressive chemotherapy, and *C. difficile* may merely be a colonizing organism; in fact, strains isolated from leukemia patients showed a variety of patterns, and some of them were nontoxigenic. However, since *C. difficile* has been recognized as a cause of colitis in leukemic patients and of colitis outbreaks in oncology wards (12, 20), an alternative explanation for our finding is that even low-virulence strains can produce disease in compromised hosts.

As found by other workers (5, 26), a pattern characteristic of *C. difficile* strains isolated from healthy neonates and children, called group 5, was also apparent. Since no strains from adult patients belong to this group, it can be speculated that these group 5 strains can be harbored only by the healthy immature intestine. One of these nontoxigenic *C. difficile* strains could be a candidate for competitive bacteriotherapy for antibiotic-associated colitis recently suggested by Seal et al. (23), but further information about the ability of these strains to colonize the adult intestine is needed.

An important subsequent step could be the electrophoretic characterization of strains isolated from healthy adult carriers; the low rate of carriage in the normal population (6) requires a long preliminary study to obtain sufficient strains.

In conclusion, the method we used is helpful and straightforward for comparing strains, e.g., in nosocomial outbreaks, without the need for sophisticated equipment or an established panel of serum samples. In fact, even if most strains isolated from individuals with PMC and AAD fall within group 2, strains isolated during an outbreak have identical protein profiles also for minor bands.

Our results indicate an association between a certain electrophoretic pattern (group 2) and virulence. If it holds true, protein electrophoresis could represent an easy method to detect a virulence marker for *C. difficile*. This could help shed light upon the clinical significance of *C. difficile* in particular situations and suggest further studies on the virulence of this organism.

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