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Application of Typing by Pulsed-Field Gel Electrophoresis to the Study of *Clostridium difficile* in a Neonatal Intensive Care Unit

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Pulsed-field gel electrophoresis (PFGE) analysis of restriction pattern polymorphism was applied to type *Clostridium difficile* isolated from neonates hospitalized in a neonatal intensive care unit, and the results were compared with those of immunoblot analysis. *C. difficile* was isolated from fecal specimens of 41 (61%) of 67 neonates during a 5-month investigation. All of these neonates were asymptomatic. Fifty-five *C. difficile* isolates from 32 patients were analyzed by PFGE after digestion with *Sma*I and *Sac*II endonucleases and by immunoblotting with 10 different antisera. Fifty-three of 55 isolates from 30 patients were identical by PFGE analysis after *Sma*I and *Sac*II digestion and immunoblot analysis. Two isolates were different from each other and from the epidemic group by both PFGE and immunoblot analysis. All 53 epidemic isolates were nontoxigenic, while the two remaining isolates were toxigenic. These results suggest the nosocomial spread of nontoxigenic *C. difficile* infection in the neonatal intensive care unit and suggest that both PFGE and immunoblot are powerful typing tools for the epidemiological study of *C. difficile*.

Clostridium difficile is well-known as a cause of pseudomembranous colitis and antibiotic-associated diarrhea. This organism is spread nosocomially (8, 12, 15), and symptomatic *C. difficile* infection requires additional treatment and a longer hospital stay. The investigation and prevention of nosocomial infection caused by *C. difficile* require the use of sensitive typing methods. Various typing methods, such as bacteriophage and bacteriocin typing (4, 20), serogrouping (9, 10), plasmid profile (8), restriction endonuclease analysis (11), and arbitrary-primer PCR (14), have been utilized in the epidemiological investigation of this organism. The typing of *C. difficile* by immunoblot with 10 different antisera was previously reported (12). Although a number of strains were not typeable because they did not react with any of the 10 antisera, the method was sensitive and useful for the investigation of a nosocomial outbreak. A limitation of the immunoblot typing which was established (12) is that new antiserum preparation is necessary when nonreactive strains are encountered.

Pulsed-field gel electrophoresis (PFGE) has been used for typing various organisms (2, 16). Digestion of chromosomal DNA with a restriction endonuclease that has infrequent DNA recognition sites results in fewer but larger fragments than can be analyzed with conventional gel electrophoresis. PFGE enables the separation of these large fragments and greatly simplifies analysis. Recently, Simor et al. used PFGE analysis and conventional restriction endonuclease analysis for the investigation of *C. difficile* recovered from a long-term-care facility and demonstrated that the comparison of digest patterns generated by PFGE was easier (18). In this paper, we evaluate PFGE analysis of *C. difficile* DNA and compare it with immunoblotting as a typing technique, using *C. difficile* isolates recovered from a neonatal intensive care unit (NICU) in Japan.

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MATERIALS AND METHODS

Bacterial strains. Ten reference strains of *C. difficile* consisting of serogroups A, B, C, D, F, G, H, I, K, and X established by Delmee et al. (9) and clinical isolates from various states in the United States were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. During a 5-month investigation (October 1985 to February 1986), 221 stool specimens were obtained from 67 neonates hospitalized in the same room of an NICU in pediatric ward of Teikyo University Hospital, Tokyo, Japan. None of these stool specimens were loose or watery. *C. difficile* was isolated and identified by standard procedures (3). Toxigenicity of isolates was determined by detecting the toxin A gene and the toxin B gene by PCR (13).

PFGE analysis. DNA extraction and PFGE were performed by a modification of the method described by Smith et al. (19). *C. difficile* was cultured in 8 ml of TYG medium (7) for 5 h anaerobically, and the cells were washed in TES buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 50 mM NaCl). The cells were resuspended in 500 μl of TES and mixed with an equal volume of 1.2% low-gelling-point agarose (FMC, Rockland, Maine) to make inserts for PFGE. The inserts were allowed to solidify in molds (Pharmacia LKB Biotechnology, Uppsala, Sweden) and incubated overnight at 50°C in lysis buffer (1.5 mg of proteinase K per ml, 10 mM Tris [pH 7.5], 0.8% Sarkosyl, 0.5 M EDTA). After inactivating the proteinase K by treatment with phenylmethylsulfonyl fluoride, DNA in the inserts was digested overnight with *Sma*I or *Sac*II (New England Biolabs Inc., Beverly, Mass.). PFGE was performed with a 1.0% agarose gel at 200 V constant voltage by the CHEF-DR II system (Bio-Rad Laboratories, Richmond, Calif.) with 25-s pulses for 3 h followed by 50-s pulses for 20 h. Gels were stained with ethidium bromide and photographed under UV light. Chromosomal DNA of *Saccharomyces cerevisiae* (Bio-Rad Laboratories) was used for molecular standard markers.

Immunoblot analysis. Immunoblotting of *C. difficile* isolates using antisera against the 10 different serogroups was performed as previously described (12). Isolates were typed to

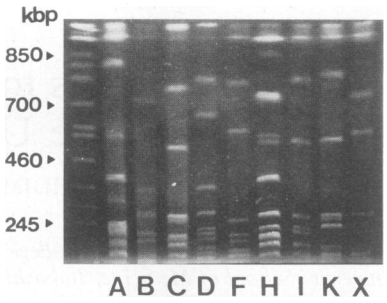

FIG. 1. PFGE patterns of *Sma*I-digested DNA from the reference strains of nine serogroups (indicated below the lanes). Each reference strain showed a distinctive restriction fragment profile. The reference strain of serogroup G could not be analyzed by PFGE because of DNA degradation. Chromosomal DNA of *S. cerevisiae* was used as size markers (far left lane).

serogroup (Delmee's grouping) (9) and subserogroup levels according to band variations in the 30- to 60-kDa range and above 60 kDa, respectively, as described previously (12).

RESULTS

Genomic DNA of the reference strains of the 10 different serogroups was analyzed by PFGE after *Sma*I digestion. Nine of the 10 reference strains showed distinctive PFGE patterns (Fig. 1). However, DNA of the reference strains of serogroup G was degraded, resulting in the strains being nontypeable. When PFGE patterns of *Sma*I-digested DNA from the reference strains of serogroups A, B, and D were compared before and after 10 subcultures, no changes in PFGE patterns were found (data not shown).

Seventy-nine (35.7%) of 221 fecal specimens from 41 (61%) of 67 neonates in the NICU were *C. difficile* culture positive (Fig. 2). For two neonates, *C. difficile* was isolated 2 days after birth. Of the 79 *C. difficile* isolates, 55 isolates from 32 neonates were available for typing and toxigenicity testing. The *Sma*I restriction patterns of 53 (96%) of 55 isolates were identical, and the isolates were designated type T-1 (Fig. 3A and Table 1). The remaining two isolates showed PFGE patterns different from those of the 53 isolates and each other and were designated types T-2 and T-3. The 53 isolates showing the same *Sma*I restriction patterns were also indistinguishable when PFGE was performed after *Sac*II digestion (Fig. 3B).

These 55 isolates from the NICU were typed by immunoblotting (Table 1). Although all 53 isolates belonging to PFGE type T-1 showed the same immunoblot pattern as the serogroup X reference strain named subserogroup X-0, the PFGE pattern of type T-1 isolates differed from that of the serogroup X reference strain (Fig. 1 and 3A and C). The PFGE type T-2 isolate showed the same immunoblot pattern as the serogroup F reference strain (serogroup F, subserogroup F-0), but they differed in PFGE patterns (Fig. 1 and 3A and C). The PFGE type T-3 isolate reacted with serogroup C antiserum, but its blotting pattern was different from that of the serogroup C reference strain (subserogroup C-0) and the isolate was designated immunoblot serogroup C, subserogroup C-2 (Fig. 3C). The type T-3 isolate and the serogroup C reference strain also showed different PFGE patterns (Fig. 1 and 3A).

The results of PCR toxigenicity testing of the 55 isolates from the NICU are summarized in Table 1. All type T-1 isolates were nontoxigenic, and both type T-2 and type T-3 isolates were toxigenic.

Ninety-one epidemiologically unrelated *C. difficile* isolates

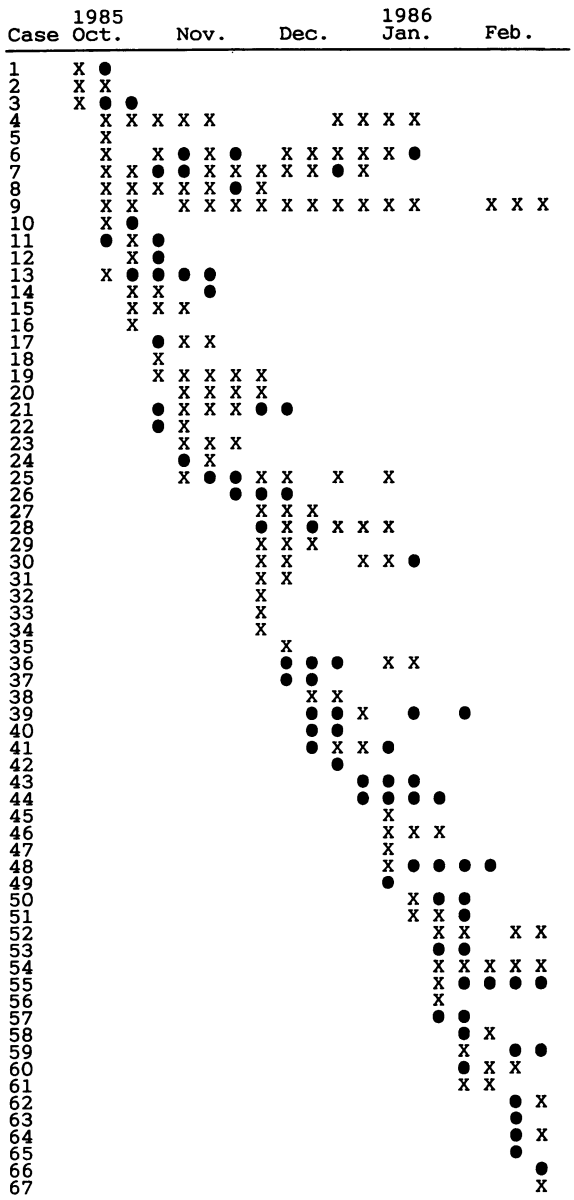

FIG. 2. Isolation of *C. difficile* from an NICU from October 1985 to February 1986. ● and X, *C. difficile*-positive and -negative feces, respectively.

were tested with both immunoblot and PFGE typing using *Sma*I digestion. Fifty-six isolates (62%) and 83 isolates (91%) were differentiated into 21 subserogroups and 52 types by immunoblot and PFGE methods, respectively. Thirty-five and eight isolates were untypeable by immunoblot and PFGE techniques, respectively. Two of the 91 isolates were untypeable either by PFGE or immunoblot analysis.

PFGE patterns of the type T-1, T-2, and T-3 strains recovered from the NICU differed from all 52 patterns of the epidemiologically unrelated isolates (data not shown).

DISCUSSION

Restriction pattern polymorphism of chromosomal DNA analyzed by PFGE has been used to type various organisms (2,

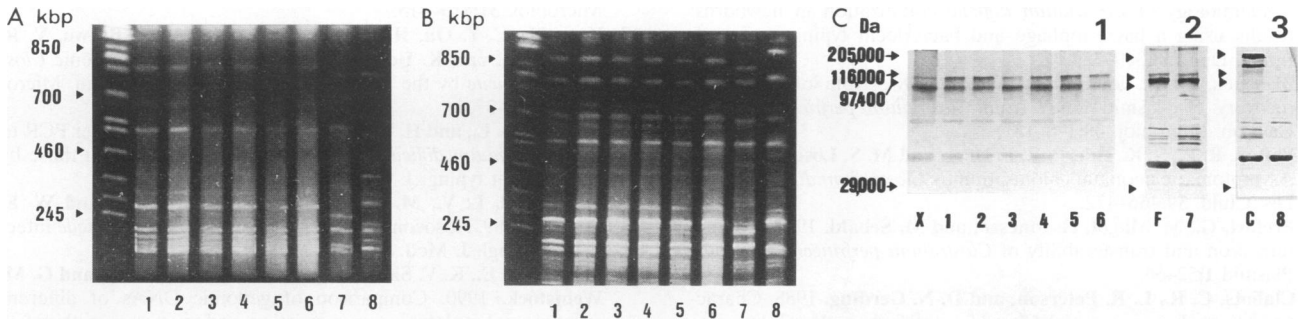

FIG. 3. Typing patterns of *C. difficile* isolates from NICU neonates (lanes 1 to 8) as determined by PFGE of *Sma*I-digested DNA (A), PFGE of *Sac*II-digested DNA (B), and immunoblot with serogroup X (C1), F (C2), and C (C3) antisera. Lanes 1 to 6, PFGE type T-1 and immunoblot type subserogroup X-0 strains; lanes 7, PFGE type T-2 and immunoblot type subserogroup F-0 strain; lanes 8, PFGE type T-3 and immunoblot type subserogroup C-2 strain; lanes X, F, and C, reference strains of serogroups X, F, and C. Chromosomal DNA of *S. cerevisiae* was used as size markers for PFGE analysis (far left lane of panels A and B).

16, 18). In this study, we used PFGE to type 55 *C. difficile* isolates from an NICU and 91 epidemiologically unrelated isolates and compared the results with those of immunoblot typing. PFGE patterns of *C. difficile* DNA after *Sma*I and *Sac*II digestion were easy to analyze because of the small number of bands produced. PFGE patterns were shown to be reproducible following 10 subcultures of *C. difficile* isolates. Distinctive PFGE profiles were demonstrated for reference strains of 9 of the 10 serogroups established by Delmee et al. (9). Despite several attempts, no PFGE pattern could be obtained for the reference strain of serogroup G because of DNA degradation during sample preparation. While eight epidemiologically unrelated isolates were untypeable by PFGE because of DNA degradation, six of them were typed as subserogroup G-0 (subserogroup of the reference strain of serogroup G) or subserogroup G-1 by immunoblotting. Strains belonging to subserogroups G-2 and G-3 were typeable by PFGE analysis. The presence of a high level of DNase activity in *Clostridium perfringens* has been reported previously (5), and nonspecific DNA degradation in restriction endonuclease analysis of *C. difficile* has been described (11). These findings suggest that *C. difficile* strains belonging to subserogroups G-0 and G-1 may have much greater deleterious DNase activity than those of other subserogroups of G and other serogroups.

The present findings of the high carriage rate (61%) of *C. difficile* in hospitalized neonates agree with previous reports (1, 10). Of 55 isolates typed, 53 (96%) were included in identical groups by both PFGE and immunoblot analysis, type T-1 and subserogroup X-0, respectively. These results suggest that the epidemic strain of *C. difficile*, typed as T-1 by PFGE and X-0 by immunoblot analysis, spread nosocomially in the NICU without causing diarrhea. The epidemic strain was shown to be nontoxigenic by a PCR assay designed to detect the toxin A and toxin B genes.

It was previously reported, for a bacteriophage and bacteriocin typing system, that a single type (20) or two types (4) were predominant among *C. difficile* isolates recovered from neonates and hospital environments. In our study, one type (immunoblot serogroup X, subserogroup X-0) was predominant. Delmee et al. (9), using the slide agglutination test, reported the recovery of nontoxigenic serogroup X strains from children.

In another report (10), 83% of *C. difficile* isolates recovered in a neonatal ward belonged to two types (toxigenic serogroup F and nontoxigenic serogroup A). The existence of nosocomial spreading and the types of epidemic strains in each hospital should reflect on the rate of neonatal colonization by *C. difficile* and the rate of recovery of toxigenic strains from neonates (1, 4, 6, 10, 17, 20).

We documented the nosocomial spread of *C. difficile* in an NICU, using both PFGE and immunoblot typing. The typing systems differ in their usefulness as typing tools. Although the PFGE typing method used in this study is more discriminatory, it has a DNA degradation problem and requires more specialized and expensive equipment than the immunoblot procedure used here. While the immunoblot procedure required the production of 10 different antisera and not all strains were typeable with these antisera, this method was able to type strains that were not typed by PFGE. The use of these two methods in combination may contribute to further knowledge concerning the epidemiology of *C. difficile* infection.

TABLE 1. PFGE and immunoblot typing results for *C. difficile* isolates from an NICU

Type as determined by:		Toxigenicity[a]		Total
PFGE	Immunoblot	Positive	Negative	
T-1	X-0		53 (30)	53 (30)
T-2	F-0	1 (1)		1 (1)
T-3	C-2	1 (1)		1 (1)
Total		2 (2)	53 (30)	55 (32)

[a] Values are numbers of isolates with numbers of patients shown in parentheses.

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