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PCR Targeted to the 16S-23S rRNA Gene Intergenic Spacer Region of *Clostridium difficile* and Construction of a Library Consisting of 116 Different PCR Ribotypes

SIMON L. J. STUBBS,[1] JON S. BRAZIER,[1]* GAEL L. O'NEILL,[2] AND BRIAN I. DUERDEN[1]

Anaerobe Reference Unit, Department of Medical Microbiology and Public Health Laboratory, University Hospital of Wales, Cardiff,[1] and Central Public Health Laboratory, London,[2] United Kingdom

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A reference library of types of *Clostridium difficile* has been constructed by PCR ribotyping isolates (n = 2,030) from environmental (n = 89), hospital (n = 1,386), community practitioner (n = 395), veterinary (n = 27), and reference (n = 133) sources. The library consists of 116 distinct types identified on the basis of differences in profiles generated with PCR primers designed to amplify the 16S-23S rRNA gene intergenic spacer region. Isolates from 55% of infections in hospitals in the United Kingdom belonged to one ribotype (type 1), but this type was responsible for only 7.5% of community infections.

Clostridium difficile is the etiologic agent of pseudomembranous colitis (PMC) and is a major cause of nosocomially acquired antibiotic-associated diarrhea (AAD) in the developed world (11). Several typing schemes have been developed to determine the relatedness of strains of *C. difficile* associated with infection, including serotyping (6, 16), immunoblotting (8), arbitrarily primed PCR (1, 15), pulsed-field gel electrophoresis (PFGE; 5, 9, 15), and PCR ribotyping (4, 7, 13). Collaborative studies have also been undertaken to assess the accordance, relative reliability, and discriminatory power of different schemes (3–5, 9, 10, 12–15). PCR ribotyping has been reported to provide a discriminatory, reproducible, and simple alternative to other typing methods (4). This technique has a number of advantages over other methods; specifically, PCR ribotyping has been shown to be more discriminatory than arbitrarily primed PCR (5) and serotyping (13) and is quicker and simpler than PFGE. PCR ribotyping has one further advantage over PFGE, since some isolates of *C. difficile* have excessive endogenous nuclease activity that renders them untypeable by PFGE (9, 10, 15). In the present study a library was constructed that comprises 116 distinct types of *C. difficile* identified on the basis of differences in amplification profiles generated by a modified PCR ribotyping technique (13). It is hoped that the library will facilitate global analysis of the epidemiology and relative virulence of strains of this nosocomial pathogen.

Bacterial isolates and PCR ribotyping. The Anaerobe Reference Unit of the Public Health Laboratory Service, based at the University Hospital of Wales in Cardiff, has provided a *C. difficile* typing service to hospitals throughout England and Wales since 1993. A modified PCR ribotyping scheme (13) has been the method of choice for typing isolates from the United Kingdom since 1995.

The identity of isolates of *C. difficile* submitted for typing was confirmed initially by the assessment of recognized phenotypic criteria (2). Enterotoxin (A) and cytotoxin (B) production were determined by the Tox A TEST immunoassay (TechLab; BioConnections, Leeds, United Kingdom) and Vero cell cytotoxicity (2), respectively.

The 2,030 isolates analyzed in the present study comprised 1,235 isolates from stool samples from hospital patients, 395 isolates from stool samples referred via community practitioners, 150 isolates from the hospital environment, 27 isolates from veterinary sources, 89 isolates from the general environment, 1 isolate from an extraintestinal human site, and 133 reference strains held in the National Collection of Type Cultures, the American Type Culture Collection and the Culture Collection, University of Göteborg, and in the personal collections of *C. difficile* types held by Delmee and others (6, 16) and other members of the International Study Group on *C. difficile* (3).

PCR ribotyping was performed in duplicate, with slight modifications to a method described previously (13). Briefly, bacteria were harvested from overnight anaerobic cultures on Fastidious Anaerobe Agar (LabM, Bury, United Kingdom) supplemented with 6% horse blood. Crude template nucleic acid was prepared by resuspension of cells in a 5% (wt/vol) solution of Chelex-100 (Bio-Rad, Hemel Hempstead, United Kingdom) and boiling for 12 min. After the removal of cellular debris by centrifugation (15,000 × g for 10 min), the superna-

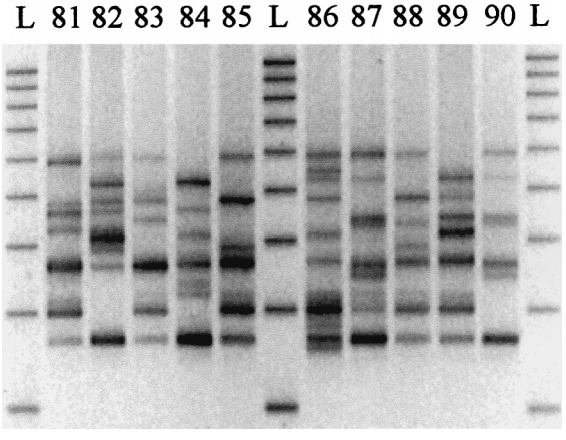

FIG. 1. PCR ribotype profiles obtained with strains belonging to PCR ribotypes 81 to 90. Lane L refers to 100-bp ladder (200 bp − 1 kbp).

* Corresponding author. Mailing address: Anaerobe Reference Unit, Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff CF4 4XW, United Kingdom. Phone: 01222 742378. Fax: 01222 742161. E-mail: Brazier@cf.ac.uk.

TABLE 1. PCR ribotypes of *C. difficile* depicting type strain, number of isolates obtained to date, and in vitro toxin production

PCR ribotype	Type strain	Production of toxin A/B	No. of isolates	Comment[a]
001	R8366	+/+	846	Serogroup G
002	R8375	+/+	60	Serogroup A_2
003	R8384	+/+	3	
004	R8386	+/+	1	
005	R8373	+/+	54	
006	R8268	+/+	11	
007	R8264	+/+	1	
008	R10568	+/+	2	
009	R8269	−/−	46	Serogroup I
010	R8270	−/−	110	Most frequent community isolate
011	R7619	+/+	4	
012	R6187	+/+	73	
013	R5252	+/+	12	
014	R11446	+/+	104	Serogroup H
015	R6685	+/+	76	Serogroup G
016	R10424	+/+	2	
017	R7404	−/+	32	Serogroup F
018	R6184	+/+	10	Serogroup A_8
019	R8637	+/+	1	
020	R10079	+/+	84	Serogroup H
021	R8763	+/+	5	Serogroup A_1
022	R4262	+/+	1	
023	R6928	+/+	43	Weak toxin expression
024	R6321	+/+	11	
025	R7276	+/+	2	
026	R10118	+/+	28	Weak toxin expression
027	R12087	+/+	1	CD196 (binary toxin strain)
028	R9300	−/−	1	
029	R8438	+/+	3	
030	R11004	−/−	8	
031	R11631	−/−	24	Serogroup K
032	R6598	−/−	2	
033	IS58	−/−	2	Serogroup E_6
034	IS81	+/+	9	Serogroup A_5
035	R11812	−/−	8	
036	CCUG20309	−/+	1	Strain 8864
037	R6641	+/+	1	
038	NCTC11206	−/−	19	Serogroup C
039	R10738	−/−	10	Serogroup A_{10}
040	R10917	−/−	1	
041	R10920	−/−	1	
042	R11817	+/+	6	
043	NCTC11382	+/+	2	
044	R10976	+/+	2	
045	R10842	+/+	4	
046	R10991	+/+	4	
047	R10541	−/+	4	
048	R10069	+/+	3	Weak toxin expression
049	R6320	+/+	8	
050	R9414	+/+	9	
051	R9549	−/−	1	
052	R6155	+/+	1	
053	IS21	+/+	6	Serogroup K
054	IS22	+/+	15	Serogroup A_1
055	R11652	+/+	3	
056	IS25	+/+	19	
057	IS27	+/+	5	Serogroup K
058	R10456	+/+	12	Weak toxin expression
059	R9304	+/+	1	
060	IS40	−/−	2	Serogroup B
061	R12099	+/+	1	
062	R11382	+/+	4	
063	IS47	+/+	2	Serogroup A_5
064	IS48	+/+	2	Serogroup A_6
065	IS49	−/−	2	Serogroup A_7
066	IS51	−/−	4	Serogroup A_9
067	IS52	−/−	4	Serogroup A_{10}
068	IS56	−/−	1	
069	IS59	−/−	1	
070	R9367	+/+	2	Serogroup K
071	IS64	−/−	1	Serogroup S_1
072	R12095	+/+	1	Serogroup X
074	IS72	−/−	1	
075	IS93	+/+	1	Serogroup A_1
076	R11548	+/+	4	Serogroup A_8
077	R10955	+/+	2	
078	R7605	+/+	13	Weak toxin expression
079	R7606	−/−	1	
081	R9764	+/+	9	
082	R7638	−/−	1	
083	R10566	+/+	1	Serogroup S_1
084	R8768	−/−	6	
085	R12098	−/−	4	Serogroup X
086	R1880	+/+	3	
087	R11840	+/+	5	
088	R10855	−/−	2	
089	R8603	−/−	1	
090	R10737	+/+	1	
091	R8643	−/−	1	
092	R10630	+/+	4	
093	R8853	+/+	1	
094	R10078	+/+	4	
095	R8858	+/+	1	
096	R9759	+/+	2	
097	R8914	+/+	1	
098	R9116	−/−	2	
099	R7425	−/−	1	
100	R12104	−/−	2	
101	R10836	+/+	1	
104	R9180	+/+	1	
106	R10459	+/+	88	
107	R9313	+/+	1	
110	R7771	−/+	2	
111	R10870	+/+	1	
112	R8631	−/−	1	
114	R11212	−/−	1	
115	R11244	+/+	6	Serogroup G
116	R11347	+/+	1	
117	R10071	+/+	1	
118	R11394	+/+	1	
119	R11805	−/−	1	
120	R11830	+/+	1	
121	R9378	−/−	1	
122	R9385	+/+	1	
123	R11907	−/−	1	
124	R11919	−/−	1	

[a] Reference where known has been made to Delmee serotype (6).

tant (10 μl) was added to a 100-μl PCR mixture containing 50 pmol of each primer 5′-CTGGGGTGAAGTCGTAACAAGG-3′ (positions 1445 to 1466 of the 16S rRNA gene) and 5′-GCGCCCTTTGTAGCTTGACC-3′ (positions 20 to 1 of the 23S rRNA gene), 2 U of *Taq* polymerase (Pharmacia), and 2.25 mM $MgCl_2$. Reaction mixtures were subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Amplification products were concentrated to a final volume of 25 μl by heating at 75°C for 105 min (13) before electrophoresis (150 mA) in 3% Metaphor agarose (FMC Bioproducts, Rockland, Maine) for 6 h at 8°C. Products were visualized by staining the gel for 20 min in ethidium bromide (0.5 μg ml^{-1}). To enable normalization of all gel patterns, a molecular size standard (100 bp; Advanced Biotechnologies, Epsom, United Kingdom) was run at five-lane intervals.

Library construction. PCR ribotype profiles were analyzed with GelCompar image analysis software (version 4.0; Applied Maths, Kortrijk, Belgium). The criterion for the proposal of a new library type was the existence of clearly discernible, reproducible (at least six profiles required per type) differences in PCR ribotype pattern from those of all other existing types. The stability, reliability, and homogeneity of the patterns con-

stituting each type have been tested with the cluster correlation algorithm with the unweighted pair group method by using arithmetic averages and fine alignment. The integrity of the library is tested routinely at monthly intervals by blind PCR ribotyping of quality control isolates. PCR ribotype profiles from routine clinical isolates are compared to those profiles which define the library by maximum matching with Pearson correlation.

Figure 1 depicts the amplification profile obtained with 10 different PCR ribotypes within the library. At present, of the 2,030 isolates of *C. difficile* typed by this method, 116 distinct PCR ribotypes have been recognized (Table 1). A representative type strain of each PCR ribotype has been stored on cryobeads (ProLab Diagnostics, Wirral, United Kingdom) and frozen at −80°C.

Strains within the library have also been analyzed by other typing schemes through international collaboration (3). The ribotyping method correlates with other typing schemes and allows subtyping of many of the types produced by other methods (Table 1) (3, 9, 13). In addition, all members of a single type have the same toxin A and toxin B production profiles, a characteristic which is not always exhibited by other typing schemes.

Routine typing of isolates of *C. difficile* from the United Kingdom. Of the isolates of *C. difficile* from patients in hospitals in the United Kingdom (n = 1,235), a single, distinct PCR ribotype (type 1) has been found to be responsible for 55% (n = 682) of all referrals to the Anaerobe Reference Unit. However, it is intriguing that PCR ribotype 1 is detected less frequently (7.5%; n = 30) among isolates referred by community practitioners (n = 395). Type 1, a subtype of serogroup G (3, 13), may be a particularly virulent or transmissible clone of *C. difficile* or may have been selected by the particular antibiotic regimens used in hospitals in the United Kingdom. Research into the possible clonality of ribotype 1 is currently being undertaken. However, PFGE analysis of PCR ribotype 1 and serogroup G is not a viable option because these isolates produce excessive nuclease activity and are untypeable by this method (9, 10). Isolates which were untypeable by PFGE have also been encountered in nosocomial outbreaks in the United States (15). The 14 isolates described by Samore et al. (15) have been included in the present study and were found to belong to PCR ribotype 1, indicating that this type may also be a potential problem in the United States.

The library contains a number of types that exhibit no toxin A activity but produce active cytotoxicity (Table 1). Some of the strains constituting these types have been isolated from individuals with active AAD or PMC and highlight the limitations of using only toxin A assays for direct detection of *C. difficile* in stool samples.

The current reference library of fully characterized PCR ribotypes seems ideal for use by groups wishing to compare the performance of other fingerprinting or typing methods and by those studying the various virulence factors attributed to *C. difficile*. It is hoped that use of the library will facilitate epidemiology and aid virulence studies on this important nosocomial pathogen.

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