Development of a Rapid and Efficient Restriction Endonuclease Analysis Typing System for *Clostridium difficile* and Correlation with Other Typing Systems

CONNIE R. CLABOTS,¹ STUART JOHNSON,² KRISTINE M. BETTIN,² PAMELA A. MATHIE,²
MAURY E. MULLIGAN,³ DENNIS R. SCHABERG,⁴ LANCE R. PETERSON,^{1,2†} AND
DALE N. GERDING^{1,2‡*}

Infectious Disease Section, Medical Service,² and Clinical Microbiology Section, Laboratory Service,¹ Minneapolis Veterans Affairs Medical Center and University of Minnesota Medical School, Minneapolis, Minnesota 55417; Infectious Disease Section, Medical Service, Long Beach Veterans Affairs Medical Center, Long Beach, California³; and University of Michigan Medical School, Ann Arbor, Michigan⁴

Received 4 January 1993/Accepted 14 April 1993

A *Hind*III restriction endonuclease analysis (REA) typing system for total genomic *Clostridium difficile* DNA including a rapid and efficient method of DNA extraction and a scheme for organizing unique electrophoretic DNA band patterns was developed. REA typing was performed by two extraction methods for 1,965 *C. difficile* isolates obtained from patients with symptomatic *C. difficile* disease, asymptomatic patients who were *C. difficile* culture positive, and environmental surfaces. This isolate collection yielded 206 unique REA types, which were organized into 75 groups. A reference strain representing each unique REA type was chosen for DNA band pattern comparisons, cytotoxin testing, and plasmid analysis. The DNA band patterns utilizing a guanidine thiocyanate-EDTA-Sarkosyl DNA extraction method were 94% reproducible, while the original and sporadically problematic diethyl pyrocarbonate-sodium dodecyl sulfate DNA extraction method was 98% reproducible when readable patterns were obtained. Reference strains from 43 of the 75 groups were cytotoxin positive, 28 groups were cytotoxin negative, and 4 groups included both toxigenic and nontoxigenic strains. Cytotoxicity of isolates with a particular REA type was always consistent with the toxicity of the reference strain for that type. REA typing was able to discriminate strain differences within types identified by the immunoblot (89 isolates), bacteriophage-bacteriocin (44 isolates), and ribotyping (23 isolates) methods. REA typing is a sensitive, discriminating, reproducible, and rapid method for differentiating *C. difficile* strains and is suitable for large-scale epidemiologic studies.

During the 1980s, several antibiotic-associated diarrhea outbreaks attributed to *Clostridium difficile* have been reported, and six different strain-typing systems have been utilized to evaluate these epidemics (7, 17, 19, 23, 29, 30). Several large outbreaks have occurred at the Minneapolis Veterans Affairs Medical Center (MVAMC) over the last 10 years, but a more prominent feature has been the elevated endemic rate of *C. difficile*-associated disease. Our research effort has focused on the manner in which patients acquire this organism and become ill. A sensitive and reproducible typing system is necessary to make accurate epidemiologic associations and elucidate the natural history of nosocomial *C. difficile* infections. Restriction endonuclease analysis (REA) of total genomic DNA was utilized in our laboratory to type *C. difficile* isolates obtained in several prospective epidemiologic studies. The purpose of this article is to describe this method in detail and to report the cataloging of REA types derived from typing approximately 2,000 *C. difficile* isolates from clinical and environmental sources. In addition, REA typing was compared with the immunoblot (IB), bacteriophage-bacteriocin (BB), and ribotyping methods for a selected group of strains.

(This work was presented in part at the 88th Annual

[Miami Beach, Fla., 8 to 13 May 1988] [16] and 91st General [Dallas, Tex., 5 to 9 May 1991] [1] meetings of the American Society for Microbiology and at the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy [Atlanta, Ga., 21 to 24 October 1990] [4a].)

MATERIALS AND METHODS

Sample collection and cultures. *C. difficile* isolates were obtained from MVAMC patients and environmental surfaces during a 10-year period, 1982 to 1991. *C. difficile* organisms isolated from loose or liquid stools of patients with diarrhea were saved by the clinical microbiology laboratory for strain typing. *C. difficile*-associated diarrhea was defined as six or more unformed stools in 36 h for a patient who had no other explanation for diarrhea, who had received antibiotics, and whose stool was *C. difficile* culture or cytotoxin positive (11). Isolates were also obtained from consenting patients (largely asymptomatic) and from the environment (bed rails, toilets, and floors beside beds and toilets) (5, 6) during three large surveillance studies on selected MVAMC wards for 9-, 40-, and 90-week periods (1a, 6, 17). Isolates from other studies focusing on nutritional and immunological status of *C. difficile* culture-positive patients as well as isolates from studies of prevention and treatment of *C. difficile* disease (18) were also saved for strain typing.

C. difficile was isolated from patient stool specimens or rectal swabs by inoculation of cycloserine-cefoxitin-fructose agar (10) and from environmental sites by using contact

* Corresponding author.

† Present address: Department of Pathology, Northwestern University Medical School, Chicago, IL 60611.

‡ Present address: Medical Service 111, Lakeside Veterans Affairs Medical Center, 333 East Huron Street, Chicago, IL 60611.

plates (RODAC; Becton Dickinson, Cockeysville, Md.) containing cycloserine-cefoxitin-fructose agar plus 1% sodium taurocholate (5). All inoculated media were incubated in an anaerobe chamber (Anaerobe Systems, Santa Clara, Calif.) for 48 h at 35°C, and *C. difficile* isolates were identified by gross colony morphology, Gram staining, and gas liquid chromatography (12). From March 1982 through December 1984, all *C. difficile* isolates were stored in chopped meat broth (Pre-Reduced II; BBL Microbiology Systems, Becton Dickinson). From January 1985 through December 1991, organisms were stored at -80°C by being suspended in tryptic soy broth plus 10% glycerol on sterile glass beads.

DNA extraction and REA. (i) **Diethyl pyrocarbonate-sodium dodecyl sulfate (DP-SDS) DNA extraction.** A single *C. difficile* colony from a 48-h anaerobic blood agar plate was inoculated into 20 ml of reduced brain heart infusion broth (Difco, Detroit, Mich.) and grown under anaerobic conditions for 18 h at 35°C. A modification of the method of Cleary et al. (8) was used for DNA extraction. Cells were washed in 10/10 TE (10 mM Tris, 10 mM EDTA [pH 8.5]), resuspended in 0.5 ml of 10-mg/ml lysozyme (Sigma, St. Louis, Mo.) in TE-sucrose (100 mM Tris, 10 mM EDTA, 25% sucrose [pH 7.0]), and incubated for 1 h at 35°C. All steps to this point were performed anaerobically. For procedures done after June 1989, the harvesting step was modified by spooling the ethanol-precipitated DNA on a glass rod, which was then washed with 70% ethanol, dried for 30 min, and solubilized in 50 µl of 10/1 TE (10 mM Tris, 1 mM EDTA [pH 7.9]).

(ii) **GES method.** Modifications of the method of Pitcher et al. (26) were made to isolate DNA from *C. difficile*. *C. difficile* isolates were grown as in the DP-SDS extraction method, except that 10 rather than 20 ml of brain heart infusion broth was required. Cells were washed in 10/1 TE, resuspended in 100 µl of 50-mg/ml lysozyme (Sigma) in 10/1 TE, and incubated for 30 min at 35°C. All steps to this point were performed anaerobically. The guanidine thiocyanate-EDTA-Sarkosyl (GES) method was incorporated in July 1991 and continued through the present time.

(iii) **Restriction endonuclease digestion.** DNA (10 to 20 µl) obtained by the DP-SDS method was incubated with 10 U of *Hind*III restriction endonuclease and digested according to the manufacturer's instructions (Promega, Madison, Wis.) with the substitution of 1 µl of 50-mg/ml nuclease-free bovine serum albumin (BSA) (Bethesda Research Laboratories, Gaithersburg, Md.) for the BSA supplied by the manufacturer. GES-extracted DNA was digested as described above, except that 20 U of *Hind*III was used and 3 µl of 100-µg/ml spermidine was added (Sigma). The restricted DNA fragments were separated by agarose gel electrophoresis (0.7% agarose) (Bethesda Research Laboratories) in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.9, with glacial acetic acid) at 50 V for 16 h. Gels were stained for 30 min in 2-µg/ml ethidium bromide, visualized by UV transillumination, and photographed with type 667 Polaroid film (21).

(iv) **REA profile interpretation.** The *Hind*III-restricted DNA band patterns for each new isolate were systematically compared with all known types, and any difference between two DNA band patterns established the isolates as separate REA types. The first isolate with a new DNA band pattern was arbitrarily designated as a reference REA type. REA types were also organized into larger groups on the basis of band pattern similarity. Similarities between the new and reference REA types were scored by visual comparison of each 1-mm segment of the top 60 mm of the DNA band patterns run on the same gel (approximate molecular weight

range, 30 to 2 kb). A similarity index was calculated as the number of identical segments expressed as a percentage of the total segments. Any new REA type with a similarity index of ≥90% compared with an existing reference REA type was included in that group. New REA types were run on the same gel with a reference REA type from one to eight groups that most closely matched the DNA band pattern of the new isolate. Any new REA type with a similarity index of <90% compared with any existing reference REA types was designated the primary reference REA type for a new group and was used for all future group comparisons. The groups were designated by letters, and distinct REA types within a group were designated by arabic numbers. A Polaroid photo of the reference type was mounted on an index card (3 by 5 in. [1 in. = 2.54 cm]) and retained for future comparisons.

Cytotoxin testing. The in vitro cytotoxicity of *C. difficile* isolates was determined by demonstration of actinomorphous changes of HEp-2 cells exposed to supernatants of chopped meat broth cultures and confirmed by neutralization with high-titer *Clostridium sordellii* antitoxin (25). Cytotoxin testing was performed initially for all reference isolates and subsequently on an additional 120 isolates, 20 isolates each from the three most prevalent cytotoxin-positive reference strains (B1, G1, and L1) and the three most prevalent cytotoxin-negative reference strains (M3, M23, and T7).

Plasmid analysis. Plasmid analysis was performed on selected *C. difficile* isolates to detect potentially confounding plasmid DNA bands within the total DNA band pattern. *C. difficile* isolates were grown in 20 ml of reduced brain heart infusion broth under anaerobic conditions for 18 h at 37°C. All manipulations were performed anaerobically until cell lysis by the method of Birnboim and Doly (2). Plasmid DNA was digested and electrophoresed as described above for the total DNA preparations. A plasmid preparation of each reference type was run on the same gel as the corresponding total genomic DNA sample. Types with identical total REA patterns except for additional plasmid bands were identified with a "p" (e.g., REA type A1p is REA type A1 plus plasmid).

Comparison typing methods. Selected MVAMC *C. difficile* isolates were tested by two phenotypic typing methods and another genotypic typing method. IB typing was performed by Maury Mulligan at the West Los Angeles VAMC in Los Angeles, Calif. (24), and BB typing was performed by Dennis Schaberg at the University of Michigan, Ann Arbor, Mich. (27).

Ribotyping was performed by a modification of the method described by Stull et al. (28). The rRNA probe was prepared by dephosphorylation of *Escherichia coli* rRNA (Sigma) with calf intestinal alkaline phosphatase (Sigma) and end labeled with [γ -³²P]ATP (New England Nuclear Research Products, Boston, Mass.). *C. difficile* DNA extraction, *Hind*III restriction enzyme digestion, and electrophoresis were performed as previously described (15), except that RNase was not added. Following electrophoresis, the *Hind*III-restricted DNA fragments were transferred to a Zetabind membrane (AMF-Cuno, Meriden, Conn.) by Southern blotting, incubated with the rRNA probe, and visualized by autoradiography.

RESULTS

From February 1982 through December 1991, 3,486 isolates were collected from patients and environmental sites at the MVAMC. Nine hundred one (26%) of the isolates were from patients with *C. difficile*-associated diarrhea, 2,098

FIG. 1. Representative isolates of six common *C. difficile* REA groups obtained from MVAMC patients are shown in lanes 2 to 7. Lane 1, lambda DNA; lanes 2 to 7, types L1 (isolate 1433), J1 (1027), G1 (1077), M1 (300), B1 (832), and Y1 (1337), respectively. Six representative types from the common REA group Y, Y1 (isolate 1337), Y2 (1459), Y3 (1701), Y4 (1323), Y6 (1172), and Y9 (1350), are shown in lanes 8 to 12, respectively. Molecular weights in thousands are shown on the left.

(60%) were from asymptomatic patients from whom *C. difficile* was cultured, and 487 (14%) were obtained from the hospital environment. Of the total 1,965 *C. difficile* isolates strain typed by REA, 520 (26%) were from patients with *C. difficile*-associated diarrhea, 993 (51%) were from asymptomatic patients from whom *C. difficile* was cultured, and 452 (23%) were from the hospital environment.

REA typing of 1,965 of the 3,486 *C. difficile* isolates yielded 206 unique REA types, which were categorized into 75 groups (Fig. 1, lanes 2 to 7). The REA types within each of the 10 most common groups compared with the primary REA type for each respective group demonstrated mean similarity indices which ranged from 94 to 98% (Fig. 1, lanes 8 to 12). The mean similarity index ($\pm$ standard error) of the primary reference REA type G1 compared with primary reference REA types from the other common groups was 78% (± 6.9) (Fig. 1, lanes 2 to 7).

Though a large number (206) of unique REA types were found, the majority of isolates (1,580 of 1,965 [80%]) were included within 15 groups. Conversely, over half (40 groups) of the groups contained four or fewer isolates (Fig. 2). The number of REA types in each group ranged from 1 to 27. In 8 of the 15 most common groups (B, G, L, E, S, N, AL, and BD), two REA types accounted for 97% (mean) of the isolates in each group. In the seven remaining common groups (M, Y, J, T, R, Z, and BM), the two most frequent REA types accounted for only 63% (mean) of the isolates.

Cytotoxicity of the reference strains correlated with the grouping of REA types in 71 of the 75 (95%) categorizations. The REA types within each of 43 groups were all cytotoxin positive and within each of 28 groups were cytotoxin negative, whereas 4 groups contained both toxigenic and nontoxigenic strains. More extensive cytotoxin testing of isolates within the most common REA types (20 isolates of each REA type) designated toxigenic (REA types B1, G1, and L1)

FIG. 2. The number of isolates obtained from *C. difficile*-positive patients (symptomatic and asymptomatic) and environmental samplings within REA groups. ■, cytotoxin-positive group; □, cytotoxin-negative group, ▨, both cytotoxin-positive and -negative group. Groups with four or fewer isolates each (36 groups [77 isolates]) are not shown. *n*, 1,965.

TABLE 1. Comparison of REA typing with IB and BB typing methods

BB type [no. of isolates]	IB type(s) [no. of isolates]	REA type	No. of REA-typed isolates in ^a :	
			BB type (39)	IB type (84)
B83/778/809/1995/33A/806 [3]	a' [2]	AO1	2	2
	Z' [3]	Y1	1	1
		Y7	0	1
		Y11	0	1
14/15/19/20/21/22;B1537 [5]	b' [2]	BR1	2	2
	g' [5]	G1	3	5
B1811/810/1481/1320/1700 [4]	c' [12]	M4	1	4
		M5	1	2
		M6	0	2
		M7	1	1
		M12	0	1
		M13	0	1
		M23	1	1
16/17 or 16 (17 and 19wk) [20]	d', d'' [42]	B1	8	26
		B2	8	10
		B4	1	1
		L1	2	3
		R9	1	1
		R10	0	1
B1811/810/1481/1320/1700/1537 [4]	e' [7 ^b]	AL1	4	4
		N1p	0	1
None (nontypeable) [2]	e'	N1	1	1
		J15	1	1
7 [1]	f' [1]	AN1	1	1
	X' [5]	E1	0	5
	Y' [5]	F2	0	5

^a The number of isolates (of the 84 typed by REA) typed by each method is given in parentheses.

^b Including isolates nontypeable by BB.

or nontoxigenic (REA types M3, M23, and T7) on the basis of reference REA type cytotoxicity gave a 100% correlation between REA types and cytotoxicity.

Plasmids were demonstrated in 99 of 206 (48%) reference strain isolates tested, but the differences in DNA band patterns were entirely accounted for by plasmid bands in only 7 of the 99 plasmid-positive reference strains. That is, alteration in organism REA type because of plasmid-derived bands in DNA band patterns occurred in only 7% of these plasmid-containing isolates.

One hundred sixty-three reference isolates were repeatedly typed by the original DP-SDS DNA extraction method, and 206 reference isolates were typed by the GES method 2 to 21 times each, with identical repeat REA patterns obtained in 98 and 94% of the cases, respectively. In the majority of cases, the disappearance or faintness of a single band accounted for minor DNA band pattern discrepancies between the two methods. The DP-SDS DNA extraction method was eventually abandoned in favor of the GES DNA extraction method because of the sporadic but repeated problems with obtaining clear REA profiles with the DP-SDS extraction method.

Isolates typed by REA were compared with IB (89 isolates) and BB (44 isolates) results. Most BB and IB typing groups encompassed several REA strain types and exhibited a one-to-one correlation to each other (Table 1). Only 5 of

89 REA-typed isolates (not included in Table 1) showed inconsistent typing results. Three isolates had REA types and IB types that were consistent with the scheme in Table 1 while the BB types were inconsistent, and two other isolates had consistent REA and BB types and inconsistent IB types.

The *E. coli* rRNA probe hybridized with 17 to 20 *Hind*III-restricted *C. difficile* DNA fragments, 13 of which were common to all *C. difficile* isolates tested (Fig. 3). In contrast, the rRNA probe hybridization pattern was markedly different for *Hind*III-restricted DNA from *Clostridium bifermens*, *Clostridium innocuum*, and *Clostridium perfringens* isolates (data not shown). Ribotyping of 16 *C. difficile* REA reference strains from our institution revealed only four ribotype patterns. The majority of the REA type strains (11 of 16) were included in one ribotype pattern (ribotype I). Seven *C. difficile* isolates from four other institutions in different states (isolate W105, Wadsworth VAMC, Los Angeles, Calif.; isolates V439, V415, and V410, Virginia Polytechnic Institute, Blacksburg; isolates 6411 and 6186, University of Michigan; and isolate 489, University of Minnesota, Minneapolis) were also analyzed by ribotyping. Ribotype I pattern isolates were identified at all four institutions, and two additional patterns were seen with two isolates from Michigan and Virginia.

FIG. 3. Representatives of the four ribotypes obtained from 16 *C. difficile* isolates, ribotype I (REA types B1, B2, B3, F1, G1, J1, J2, J10, Y6, and BM1p), ribotype II (E1, M1, and M4), ribotype III (N1p), and ribotype IV (P1), are shown in lanes 1 to 4, respectively.

DISCUSSION

Numerous typing methods have been employed by investigators in the continuing effort to evaluate nosocomial *C. difficile* epidemiology. Typing methods that have been examined include BB (27), polyacrylamide gel electrophoresis after incorporation of [³⁵S]methionine (PAGE-[³⁵S]) (29), IB (24), serogrouping (30), plasmid profile (7), ribotyping (16), and REA of total genomic DNA (9, 20).

Our laboratory originally employed plasmid isolation as an epidemiologic typing method for *C. difficile* (7). However, we recovered plasmids in fewer than half of the isolates, a result limiting the usefulness of this method. Therefore, we developed a large-scale REA typing method (15), similar to that used by several other investigators (9, 20), which utilized whole genomic DNA from the organism. This large-scale REA typing method involved large reagent volumes and proved too time-consuming for potential typing of several thousand strains; therefore, more-rapid methods were explored. Rapid DNA extraction methods involving small reagent volumes (<1.5 ml) have allowed us to use REA typing for large epidemiologic studies. A modification of the GES DNA extraction method of Pitcher et al. (26) has proven to be fast and efficient in isolating DNA from *C. difficile* after our initial experience with a rapid but sometimes problematic DP-SDS DNA extraction method.

Sporadic problems with the DP-SDS method of DNA extraction from *C. difficile* were manifested by undigestible DNA and unreadable DNA band patterns. Several properties of *C. difficile* are likely involved in the difficulties in extracting DNA and obtaining clear REA DNA band patterns. First, nuclease activity was found to be high in other clostridia (3) and was postulated by Devlin et al. (9) to be the cause of nonspecific background staining in agarose gels of restriction enzyme-digested *C. difficile* DNA. DNA band pattern clarity was much improved with the use of the GES DNA extraction method possibly because of the efficiency of guanidine thiocyanate in inhibiting nucleases (4). The problems we encountered upon repeat restriction digestion of stored DNA samples using the DP-SDS extraction method may also reflect a high level of nuclease contamination.

Second, *C. difficile* was relatively resistant to lysis, resulting in small amounts of DNA for REA typing. Increasing the volume of cell broth culture approximately 10-fold with methods originally developed for *Enterobacteriaceae* or staphylococcal strains alleviated this problem. Devlin et al. (9) also found lysis of *C. difficile* to be difficult and recommended the addition of 1.6% glycine to the growth media to precondition the cells for more complete lysis.

Finally, excessive protein interference may also impede efficient DNA extraction from *C. difficile*. A large amount of protein residue just prior to sample rehydration and restriction digestion, possibly due to the larger culture volume used and the nature of gram-positive bacteria (13), was observed during our attempts to extract *C. difficile* DNA. Removal of this protein was achieved by addition of phenol to the chloroform-2-pentanol extraction mixture with a full minute of manual agitation with the GES extraction method and by DNA spooling with the DP-SDS extraction method.

Determining whether DNA band patterns were identical or different from reference REA types was not difficult, although the large number of bands within the patterns initially appeared complex. Viewing the DNA band patterns as a whole and application of the mm segment scoring system for grouping close matches helped simplify the otherwise complex DNA band pattern analysis. While it is likely that isolates with different DNA band patterns within the same group are more closely related than isolates not within the same group, it is difficult to quantitate their relatedness. We have required identical DNA band patterns to establish clonality when making specific epidemiologic associations rather than using our more broad group designations. Different REA types from within the same group have rarely been found in potentially linked epidemiologic samples, such as those from two infected patients in the same room, those from an infected patient and his environment, and serial cultures from the same patient. Grouping DNA band patterns by similar overall patterns was begun to help simplify the identification of new, unknown isolates, but we have been cautious about using group designations for more than a broad epidemiologic data overview.

Ribotype analysis of *C. difficile* strains allowed for easier pattern comparisons between strains due to the relatively few DNA fragments which hybridized with the *E. coli* rRNA probe. However, the discriminatory power of this method was clearly below that of REA typing, as most of the REA type reference strains were included in one ribotype pattern. Further modifications of this method such as use of two restriction enzymes prior to hybridization could potentially increase its sensitivity (14). Ribotyping was also a lengthier process than REA typing and hence less suitable for typing large numbers of isolates.

We, as did Devlin et al. (9), found REA typing to be consistent with the ability of a strain to produce cytotoxin not only among isolates within the same REA type but also between isolates with overall similar REA patterns grouped by our system. Variation in cytotoxin production by isolates of the same type with the phenotypic methods of PAGE-[³⁵S] (31), IB (22), and serogrouping (30) has been reported.

Typing method comparisons together with toxin production results suggest that REA typing is more discriminating than ribotyping and most phenotypic methods in detecting strain differences. Comparisons of *C. difficile* strains typed by IB, BB, REA, and ribotyping showed that most BB, IB, and ribotyping groups encompassed several REA types or groups. More epidemiologic linkages are likely when a typing system with fewer strain type discriminations than

those found with REA typing is used, and therefore epidemiologic conclusions may vary or conflict depending on the typing system in use.

REA typing using the GES extraction method is a rapid, discriminating, and reproducible method for differentiating *C. difficile* strains and is applicable for large-scale epidemiologic investigations.

ACKNOWLEDGMENTS

This study was supported by the Department of Veterans Affairs.

We thank Terrence L. Stull for his advice in adapting the ribotyping method and Patrick Cleary for his contributions to the development of the REA method.

REFERENCES

- Bettin, K., C. Clabots, S. Johnson, L. Peterson, and D. Gerding. 1991. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991, abstr. L-35.
- Bettin, K. M., S. Kolb, C. Clabots, and D. Gerding. 1990. Frequency of introduction and acquisition of *Clostridium difficile* by patients in private versus multibed rooms. Clin. Res. 38:870A.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
- Blaschek, H. P., and M. A. Klacik. 1984. Role of DNase in recovery of plasmid DNA from *Clostridium perfringens*. Appl. Environ. Microbiol. 48:178-181.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299.
- Clabots, C. R., K. M. Bettin, S. Johnson, M. E. Mulligan, D. R. Schaberg, L. R. Peterson, and D. N. Gerding. 1990. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1031.
- Clabots, C. R., K. M. Bettin, L. R. Peterson, and D. N. Gerding. 1991. Evaluation of cycloserine-cefoxitin-fructose agar and cycloserine-cefoxitin-fructose broth for recovery of *Clostridium difficile* from environmental sites. J. Clin. Microbiol. 29:2633-2635.
- Clabots, C. R., S. Johnson, M. M. Olson, L. R. Peterson, and D. N. Gerding. 1992. Acquisition of *Clostridium difficile* by hospitalized patients: evidence for colonized new admissions as a source of infection. J. Infect. Dis. 166:561-567.
- Clabots, C. R., L. R. Peterson, and D. N. Gerding. 1988. Characterization of a nosocomial *Clostridium difficile* outbreak by using plasmid profile typing and clindamycin susceptibility testing. J. Infect. Dis. 158:731-735.
- Cleary, P. P., E. L. Kaplan, C. Livdahl, and S. Skjold. 1988. DNA fingerprints of *Streptococcus pyogenes* are M type specific. J. Infect. Dis. 158:1317-1323.
- Devlin, H. R., W. Au, L. Foux, and W. C. Bradbury. 1987. Restriction endonuclease analysis of nosocomial isolates of *Clostridium difficile*. J. Clin. Microbiol. 25:2168-2172.
- 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.
- Gerding, D. N., M. M. Olson, L. R. Peterson, D. G. Teasley, R. L. Gebhard, M. L. Schwartz, and J. T. Lee, Jr. 1986. *Clostridium difficile*-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. Arch. Intern. Med. 146:95-100.
- Holdeman, L. V., W. E. C. Moore, and E. P. Cato (ed.). 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
- Ingraham, J. L., O. Maaloe, and F. C. Neidhardt (ed.). 1983. Growth of the bacterial cell, p. 26. Sinauer Associates Inc., Sunderland, Mass.
- Izard, N. C., H. Hachler, M. Grehn, and F. H. Kayser. 1992. Ribotyping of coagulase-negative staphylococci with special emphasis on intraspecific typing of *Staphylococcus epidermidis*. J. Clin. Microbiol. 30:817-823.
- Johnson, S., A. Adelman, C. R. Clabots, L. R. Peterson, and D. N. Gerding. 1989. Recurrences of *Clostridium difficile* diarrhea not caused by the original infecting organism. J. Infect. Dis. 159:340-343.
- Johnson, S., C. Clabots, D. Gerding, and L. Peterson. 1988. Comparison of three molecular analytic methods for the epidemiologic study of *Clostridium difficile*, abstr. L-10. Abstr. Annu. Meet. Am. Soc. Microbiol. 1988.
- Johnson, S., C. R. Clabots, F. V. Linn, M. M. Olson, L. R. Peterson, and D. N. Gerding. 1990. Nosocomial *Clostridium difficile* colonization and disease. Lancet 336:97-100.
- Johnson, S., S. R. Homann, K. M. Bettin, J. N. Quick, C. R. Clabots, L. R. Peterson, and D. N. Gerding. 1992. Treatment of asymptomatic *Clostridium difficile* carriers (fecal excretors) with vancomycin, Metronidazole, or placebo. Ann. Intern. Med. 117:297-302.
- Kaatz, G. W., S. D. Gittlin, D. R. Schaberg, K. H. Wilson, C. A. Kauffman, S. M. Seo, and R. Fekety. 1988. Acquisition of *Clostridium difficile* from the hospital environment. Am. J. Epidemiol. 127:1289-1294.
- Kuijper, E. J., J. H. Oudbier, W. N. H. M. 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.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- McFarland, L. V., G. W. Elmer, W. E. Stamm, and M. E. Mulligan. 1991. Correlation of immunoblot type, enterotoxin production, and cytotoxin production with clinical manifestations of *Clostridium difficile* infection in a cohort of hospitalized patients. Infect. Immun. 59:2456-2462.
- 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.
- 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.
- Peterson, L. R., J. J. Holter, C. J. Shanholtzer, C. R. Garrett, and D. N. Gerding. 1986. Detection of *Clostridium difficile* toxins A (enterotoxins) and B (cytotoxin) in clinical specimens. Evaluation of a latex agglutination test. Am. J. Clin. Pathol. 86:208-211.
- Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8:151-156.
- Sell, T., D. R. Schaberg, and F. R. Fekety. 1983. Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. J. Clin. Microbiol. 17:1148-1152.
- Stull, T. L., J. J. LiPuma, and T. D. Edlund. 1988. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J. Infect. Dis. 157:280-296.
- Tabaqchali, S., S. O'Farrell, D. Holland, and R. Silman. 1984. Typing scheme for *Clostridium difficile*: its application in clinical and epidemiological studies. Lancet i:935-938.
- Toma, S., G. Lesiak, M. Magus, H. L. Lo, and M. Delmee. 1988. Serotyping of *Clostridium difficile*. J. Clin. Microbiol. 26:426-428.
- Wren, B. W., S. R. Heard, and S. Tabaqchali. 1987. Association between production of toxins A and B and types of *Clostridium difficile*. J. Clin. Pathol. 40:1397-1401.