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Comparison of DNA Fingerprinting Methods for Use in Investigation of Type E Botulism Outbreaks in the Canadian Arctic

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Pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, and automated ribotyping were compared for epidemiological typing of *Clostridium botulinum* type E using clinical and food isolates associated with four botulism outbreaks occurring in the Canadian Arctic. All type E strains previously untypeable by PFGE, even with the use of a formaldehyde fixation step, could be typed by the addition of 50 μM thiourea to the electrophoresis running buffer. Digestion with SmaI or XhoI followed by PFGE was used to link food and clinical isolates from four different type E botulism outbreaks and differentiate them from among 39 group II strains. Strain differentiation was unsuccessful with the automated ribotyping system, producing a single characteristic EcoRI fingerprint common to all group II strains. RAPD analysis of *C. botulinum* group II strains was not consistently reproducible with primer OPJ-6 or OPJ-13, apparently discriminating between epidemiologically related strains. A modified PFGE protocol was judged to be the most useful method for typing epidemiologically related *C. botulinum* type E strains, based on its ability to type all strains reproducibly and with an adequate level of discrimination.

Food-borne botulism remains a serious public health concern in Arctic regions, where the disease is endemic (3–5, 46) and is associated with the consumption of contaminated native foods, especially aged marine mammal meat (52). Most outbreaks have been caused by *Clostridium botulinum* type E, with rare cases attributed to type A and type B strains (7). Although not confirmed by source-tracking methods, the source of contamination of butchered meat from harvested seal, walrus, and beluga whale is presumed to be from *C. botulinum* spores present in sediment, shoreline soil, or the surfaces of coastal rocks (38–39).

The species *C. botulinum* is divided into four physiological groups (I to IV) producing seven types of botulinum neurotoxin (A to G) based on the antigenic specificity of the toxin produced (6). Only serotypes from group I (A, B, and F) and group II (B, E, and F) cause human illness (6). Fingerprinting methods such as pulsed-field gel electrophoresis (PFGE) (21–22) and randomly amplified polymorphic DNA (RAPD) typing (29) in combination with PFGE have been used to study the genetic relatedness of *C. botulinum* type E strains isolated from fish, fishery products, and sediments. PFGE and RAPD typing have revealed extensive genetic biodiversity among type E isolates (22, 29). PFGE has recently been applied for typing outbreak strains of *C. botulinum* group I (20), but its usefulness during a food-borne outbreak investigation remains unknown, in part because of the low incidence of botulism outbreaks yielding isolates from clinical, food, and environmental sources (33).

Type E strains belonging to *C. botulinum* group II have previously been reported to be more difficult to type by PFGE due to the degradation of DNA during the procedure (21). The untypeable group II strains presumably produced high levels of extracellular DNase, causing DNA degradation during their isolation (21). The use of a large number of cells generated in broth culture, combined with a step in which harvested cells were fixed in formaldehyde, resolved the problem of smearing observed during PFGE of many *C. botulinum* group II strains (21). However, it was estimated that about 10% of type E strains could not be typed by PFGE in spite of the formaldehyde step (22, 24) and that RAPD (29) and manual ribotyping (23) were suitable alternative typing methods. An automated ribotyping system (RiboPrinter) has been evaluated for typing *C. botulinum* group I and group II strains, but only a few type E strains could be typed using this method (45).

The problem of DNA degradation affecting the typeability of PFGE is not exclusive to *C. botulinum*; it also occurs in other clostridial species, including *C. perfringens* (36), *C. sporogenes* (35), and *C. difficile* (34) as well as several gram-negative bacteria (30, 43, 44). The addition of thiourea to the running buffer resolved the smearing problem for typing some *C. difficile* isolates (14), but it required additional modifications of the PFGE protocol to reduce DNA degradation (2, 19). Klaassen et al. (32) concluded that amplified fragment length polymorphism analysis, which is not affected by DNA degradation, was an acceptable method that overcame the limitations seen with PFGE. Amplified fragment length polymorphism analysis has recently been used for the characterization of *C. botulinum* group I and II strains, resulting in all strains, including those from group II, being typeable (31).

The high incidence of food-borne botulism in the Nunavik region (northern Quebec, Canada) of approximately five cases per 10,000 people annually presented an opportunity to compare the performances of PFGE with RAPD analysis and automated ribotyping using epidemiologically related clinical and food isolates. In this study, we compared the three methods in terms of typeability, reproducibility, discriminatory power, and

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TABLE 1. *Clostridium botulinum* group II strains used in this study

Serotype and strain	Origin	Location	Yr of isolation	Source[a]
Serotype B				
2B	Marine sediment	Pacific Coast, United States	1960s	Eklund/Solomon
17B	Marine sediment	Pacific Coast, United States	1960s	Eklund/Solomon
DB-2	Marine sediment	Pacific Coast, United States	1968	Eklund/Solomon
II60-15B	Feces	British Columbia, Canada	1983	BRS
KAP-B-3	Kapchunka	California, United States	1981	Solomon
KAP-B-8	Kapchunka	California, United States	1981	Solomon
Serotype E				
Russ	Sturgeon intestine	Russia	1935	Gunnison/NR
8550	Perch intestine	France	1951	Prévot/NR
Gordon	Clinical specimen	Quebec, Canada	1975	Gauvreau
Bennett	Gastric liquid	Happy Valley, Canada	1976	BRS
FE9507EEA	Feces	Kangiqsualujjuaq, Canada	1995	BRS
MI9507E	Misiraq	Kangiqsualujjuaq, Canada	1995	BRS
F9508EMA	Feces	Kuujjuaq, Canada	1995	BRS
VI9508E	Seal meat	Kuujjuaq, Canada	1995	BRS
FE9508EPB	Feces	Tasiujaq, Canada	1995	BRS
S9510E	Seal meat	Kuujjuaq, Canada	1995	BRS
GA9709EHS	Gastric liquid	Kangiqsualujjuaq, Canada	1998	BRS
GA9709ENS	Gastric liquid	Kangiqsualujjuaq, Canada	1998	BRS
GA9709EJA	Gastric liquid	Kangiqsualujjuaq, Canada	1998	BRS
FE9709ELB	Feces	Kangiqsualujjuaq, Canada	1998	BRS
FE9709EBB	Feces	Kangiqsualujjuaq, Canada	1998	BRS
FE9909ERG	Feces	Inuvik, Canada	1999	BRS
FE0005EJT	Feces	Inuvik, Canada	2000	BRS
MU0005EJT	Muktuk	Inuvik, Canada	2000	BRS
SW280E	Saltwater	Koksoak River, Canada	2001	This study
SO309E2	Shoreline soil	Hudson Bay, Canada	2001	This study
SO325E1	Shoreline soil	Ungava Bay, Canada	2001	This study
SO326E1	Shoreline soil	Ungava Bay, Canada	2001	This study
SO329E1	Shoreline soil	Ungava Bay, Canada	2001	This study
SP455456E2	Coastal rock	Ungava Bay, Canada	2001	This study
SOKR-23E1	Marine sediment	Koksoak River, Canada	2002	This study
SOKR-29E1	Shoreline soil	Ungava Bay, Canada	2002	This study
SOKR-37E2	Freshwater sediment	Koksoak River tributary, Canada	2002	This study
Serotype F				
70F	Marine sediment	California coast, United States	1960s	Eklund/Solomon
190F	Marine sediment	California coast, United States	1960s	Eklund/Solomon
202F	Marine sediment	California coast, United States	1965	Eklund/Solomon
205F	Marine sediment	California coast, United States	1960s	Eklund/Solomon
610F	Salmon	Columbia River, Oregon	1966	Craig/Solomon
19501F	Marine sediment	Oregon coast, United States	1960s	Eklund/Crowther

[a] In entries with a slash, the first name is the initial source, and the second is the donor source. H. Solomon, U.S. Food and Drug Administration, Washington, D.C.; BRS, Botulism Reference Service for Canada, Health Canada, Ottawa, ON, Canada; NR, no records of the donor source found; Prévot, see the work of Prévot and Huet (40); L. Gauvreau, Laboratoire de microbiologie, Centre hospitalier de l'Université Laval, Ste.-Foy, QC, Canada; this study, ecological survey of *Clostridium botulinum* type E in Nunavik, Canada; J. S. Crowther, Unilever Research, Sharnbrook, Bedford, England.

epidemiological concordance and evaluated whether the addition of thiourea to the running buffer could improve the typeability of *C. botulinum* group II strains using PFGE.

MATERIALS AND METHODS

Bacterial strains and growth media. Thirty strains of *C. botulinum* group II from the culture collection of the Botulism Reference Service for Canada and nine strains of *C. botulinum* type E recently isolated from the Arctic environment were used in this study. The origin, source, and date of isolation of all strains are described in Table 1. Nine environmental isolates and 12 food or clinical isolates of *C. botulinum* type E showing smearing during PFGE were selected to evaluate the protective effect of thiourea. Eight clinical and three food isolates associated with four type E botulism outbreaks in the Canadian Arctic were included to evaluate the ability of each typing method to link outbreak isolates. The outbreaks were not related and occurred in three separate Inuit communities at different time periods between July 1995 and May 2000. In addition to these isolates, 16 *C. botulinum* type B, E, and F strains from group II of various origins were included to assess the discriminatory power of each typing method.

Isolates were stored at −86°C on Microbank beads (Pro-Lab Diagnostics, Richmond Hill, Canada) and were routinely grown on Bacto McClung-Toabe agar base (Becton, Dickinson and Company, Sparks, Maryland) containing 0.5% Bacto yeast extract (Becton, Dickinson and Company, Sparks, Maryland) and 5% egg yolk suspension (two egg yolks in 100 ml of 0.85% saline solution) for 2 to 3 days in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at room temperature. For PFGE and RAPD analyses only, one whole colony from each isolate was transferred into 8 ml of SPGY broth composed of 5% special peptone (Oxoid Ltd., Basingstoke, England), 0.5% Bacto peptone (Becton, Dickinson and Company, Sparks, Maryland), 2% Bacto yeast extract (Becton, Dickinson and Company, Sparks, Maryland), 0.4% glucose, and 0.1% sodium thioglycolate with a pH of 7.2 for overnight growth using the same temperature and an anaerobic atmosphere as described above. Under these growth conditions, the optical density at 540 nm was 1.2 ± 0.2, corresponding to 10^6 to 10^7 CFU per ml, following overnight incubation.

PFGE analysis. The PFGE procedure combined the method of Hielm et al. (21) and the short DNA preparation protocol of Sperner et al. (47), with some modifications. Cultures grown overnight in 8 ml of SPGY were chilled on ice, and cells were harvested by centrifugation at 14,500 × *g* for 10 min at 4°C. Cells were suspended in 960 μl of PIV buffer (10 mM Tris, 1 M NaCl, pH 7.5) and fixed with formaldehyde (4%, vol/vol) to reduce the endogenous DNase activity. Cells were washed twice in 1 ml of PIV buffer, harvested by centrifugation at 16,000 × *g* for 5 min, and resuspended in 1 ml of lysis buffer (6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, and 0.5% sodium lauryl sarcosine, pH 7.6) containing RNase A (20 μg/ml), lysozyme (1 mg/ml), and mutanolysin (20 U/ml), which were added to the suspensions just prior to the embedding of cells in 2% low-melting-point agarose (Seakem Gold agarose; BMA, Rockland, Maine). The agarose plugs, made from a mixture of equal volumes (500 μl) of cell suspension and 2% low-melting-point agarose, were incubated in 3 ml of the same lysis solution at 37°C for 2 h with gentle shaking and then placed in 2 ml of ESP solution (0.5 M EDTA and 10% sodium lauryl sarcosine, pH 8.0) containing 150 μg proteinase K per ml for 2 h at 50°C with gentle shaking. Plugs were washed five times in 15 ml of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.6) at 37°C for 15 min and stored in TE at 4°C until use.

The DNA embedded in agarose was digested with a high concentration of restriction enzymes to reduce the incubation time. Two plugs were restricted with 200 U of SmaI (Roche Diagnostics GmbH, Mannheim, Germany) or 200 U of XhoI (Roche Diagnostics GmbH, Mannheim, Germany) in 150 μl of the corresponding enzyme buffer and incubated at 25°C for 2 h or at 37°C for 2 h, respectively. Restriction fragments were separated in a 1% low-melting-point agarose (Seakem Gold agarose; BMA, Rockland, Maine) gel in 0.5× Tris-borate-EDTA (45 mM Tris-borate, 1 mM EDTA) (Sigma, St. Louis, Missouri), with and without 50 μM thiourea, in a contour-clamped homogeneous electric field MAPPER apparatus (Bio-Rad Laboratories, Hercules, California) set for 6.0 V/cm at a temperature of 10°C for 16 h using initial and final pulse times of 1 and 18 s, respectively. A low-range PFGE molecular weight marker (New England BioLabs, Ipswich, Massachusetts) was used as a fragment size marker. The gels were stained using ethidium bromide (1 mg/liter) for 30 min, destained in distilled water for 60 min, and photographed using a Gel-Doc 1000 system (Bio-Rad Laboratories, Hercules, California). Two isolates per strain were grown and tested at different times by PFGE with both restriction enzymes for quality control and to assess the reproducibility of the procedure.

Automated ribotyping. Ribotyping analyses were conducted with an automated RiboPrinter system (DuPont Qualicon, Wilmington, Delaware) operated with proprietary preparations and membrane-processing packs, which include reagents for cell lysis, deproteination, restriction digestion, and hybridization. A complete description of the RiboPrinter system from cell lysis to image analysis was previously reported (10, 11). Two restriction enzymes, EcoRI (Qualicon, Wilmington, Delaware) and ClaI (Roche Diagnostics GmbH, Mannheim, Germany), were selected based on previous reports (23, 45), while PstI (Qualicon, Wilmington, Delaware) was tested with *C. botulinum* for the first time. The temperature and time for restriction digestion were set at 37°C for 120 min for ClaI, while the standard protocols for EcoRI and PstI were followed. Replicate samples of group II strains were also included within runs for quality control.

RAPD analysis. DNA isolation and RAPD analysis were essentially performed as described by Hyytiä et al. (28), with minor modifications. Cells in 8 ml of SPGY broth were harvested by centrifugation at 1,500 × *g* and 4°C for 30 min, washed in 5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5), and resuspended in 500 μl of TE containing lysozyme (8.3 mg/ml) and mutanolysin (167 IU/ml) following low-speed centrifugation. Lysis was initially performed by incubating the cell suspensions at 37°C with gentle shaking for 2 h, after which incubation was continued for 1 h at 60°C with the addition of proteinase K (50 μg/ml), EDTA (9.5 mM), NaCl (0.24 M), and sodium dodecyl sulfate (0.8%). A phenol-chloroform-isoamyl alcohol (25:24:1) extraction was then performed to remove proteins from the cell lysate (1:1, vol/vol), and DNA was recovered in the aqueous phase after centrifugation at 16,000 × *g* for 5 min. A subsequent extraction was performed with phenol-chloroform-isoamyl alcohol (25:24:1) to remove any residual proteins, and DNA was precipitated overnight with two volumes of 95% ice-cold ethanol at −20°C. The solution was centrifuged at 16,000 × *g* for 20 min and air dried, and then the precipitate was resuspended in TE. To remove any RNA present in the extract, RNase A (166 μg/ml) was first heat treated to remove any DNase, added into the solution, and then incubated with gentle shaking at 37°C for 40 min. For better precipitation, sodium chloride (0.21 M) was added prior to performance of a third phenol-chloroform-isoamyl alcohol (25:24:1) extraction. DNA was again precipitated with 2 volumes of 95% ice-cold ethanol maintained at −20°C for 1 h, followed by a 70% ethanol wash. The purified DNA was resuspended in 100 μl of Tris buffer (10 mM Tris, pH 7.5), quantified with a UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and diluted to a final concentration of 5 ng/μl.

RAPD analysis was performed using Ready-To-Go RAPD analysis beads (Amersham Biosciences, Piscataway, New Jersey), which are coated with the required PCR reagents at defined concentrations, except for the primer. Sample preparation was performed according to the manufacturer's instructions using 10 ng of genomic DNA and 25 pmol of a single primer mixed with an RAPD bead in a 25-μl reaction volume. Two previously tested arbitrary primers (28, 29), OPJ-6 (5′-TCGTTCCGCA) and OPJ-13 (5′-CCACACTACC) (Operon Biotechnologies, Inc., Huntsville, Alabama), were used with the following cycling parameters. The mixture was subjected to an initial denaturing step at 95°C for 5 min, followed by 45 cycles of 1 min at 95°C, 1 min at 36°C, 2 min at 72°C, and a final extension step for 5 min at 72°C, using a Tpersonal thermocycler (Biometra, Goettingen, Germany). The amplified products were separated by electrophoresis in a 2% agarose gel using 1× Tris-borate-EDTA (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA) buffer containing 0.5 μg/ml of ethidium bromide at 100 V for 2 h (Mini Gel electrophoresis system gel X_{Lplus}; Labnet International Inc., Woodbridge, New Jersey). Two positive controls (*Escherichia coli* strains provided in the Ready-To-Go RAPD kit) and a negative control (a reaction tube containing PCR reagents and a primer but no DNA) were used for each set of samples. The DNA molecular weight marker VI (154 to 2176 bp) (Boehringer Mannheim GmbH, Mannheim, Germany) was used as the size marker. The reproducibility of the method was tested by analyzing two isolates for each strain cultured and tested at different times.

Fingerprint analysis. Comparisons of DNA fragment patterns and cluster analysis were performed using the BioNumerics version 3.5 software (Applied Maths, Kortrijk, Belgium). The level of similarity among DNA fragment patterns was estimated using the Dice coefficient correlation, and cluster analysis of fingerprints was performed with the unweighted-pair group method using arithmetic averages (UPGMA). Both the optimization and position tolerance were set at 1% for all pairwise and cluster analyses, based on the analysis of DNA fragment patterns of duplicate samples tested by PFGE and automated ribotyping. Low fragment sizes (<23.1 kb) generated by SmaI digestions were ignored in the analysis. Simpson's index of diversity was used to evaluate the discriminatory ability of the typing methods (25).

RESULTS

PFGE analysis. Of the 39 *C. botulinum* group II isolates analyzed with the modified PFGE protocol, only one type B strain (DB-2) could not be typed due to the low DNA yield available for restriction. To overcome this problem, cells from three separate cultures of DB-2 were then combined and fixed with formaldehyde directly in SPGY broth at room temperature and harvested at a lower centrifugation speed. These modifications increased DNA yield and allowed typing of DB-2. All DNA of *C. botulinum* group II strains was successfully cleaved with SmaI and XhoI, but the resolution of DNA macrorestriction patterns of several type E strains was thiourea dependent. When thiourea was not included in the electrophoresis buffer, smearing of the PFGE lane was observed with specific type E strains only and not with type B or F strains belonging to group II. For most of these type E strains, DNA smearing occurred between the markers at 9.4 and 294 kb (Fig. 1a). Adding thiourea (50 μM) to the running buffer resolved (Fig. 1b) the smearing of the 21 untypeable type E isolates and did not alter the DNA macrorestriction patterns of the two type E strains included as controls (i.e., typeable without thiourea). Mock digestion without enzymes followed by PFGE was performed with embedded DNA from six untypeable type E isolates, with the DNA remaining intact after an overnight incubation at 37°C, suggesting that the degradation occurs during electrophoresis when thiourea is not added (data not shown). Elimination of the formaldehyde fixation step during DNA isolation of 12 group II strains caused smearing in PFGE

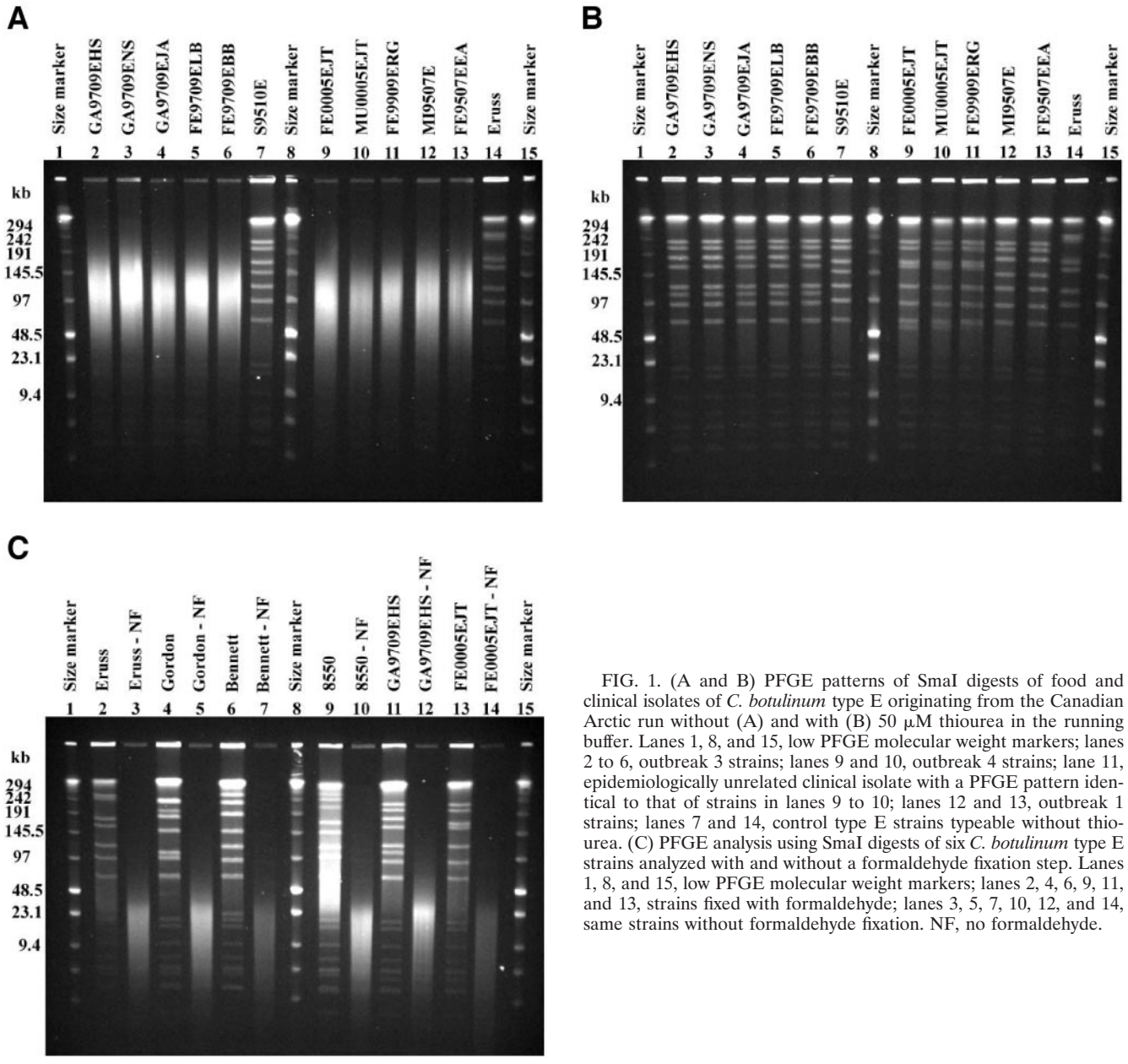

FIG. 1. (A and B) PFGE patterns of SmaI digests of food and clinical isolates of *C. botulinum* type E originating from the Canadian Arctic run without (A) and with (B) 50 μM thiourea in the running buffer. Lanes 1, 8, and 15, low PFGE molecular weight markers; lanes 2 to 6, outbreak 3 strains; lanes 9 and 10, outbreak 4 strains; lane 11, epidemiologically unrelated clinical isolate with a PFGE pattern identical to that of strains in lanes 9 to 10; lanes 12 and 13, outbreak 1 strains; lanes 7 and 14, control type E strains typeable without thiourea. (C) PFGE analysis using SmaI digests of six *C. botulinum* type E strains analyzed with and without a formaldehyde fixation step. Lanes 1, 8, and 15, low PFGE molecular weight markers; lanes 2, 4, 6, 9, 11, and 13, strains fixed with formaldehyde; lanes 3, 5, 7, 10, 12, and 14, same strains without formaldehyde fixation. NF, no formaldehyde.

lanes, regardless of serotype and inclusion of thiourea in the running buffer (Fig. 1c).

Pairwise analysis of replicates showed that PFGE was reproducible and that only on rare occasions did some replicates differ by the presence or absence of a faint fragment. Cluster analysis of SmaI and XhoI PFGE patterns generated dendrograms characterized by a large cluster of type E strains, which diverged from a cluster composed of type B and type F strains at 61% and 55% similarity coefficients, respectively (Fig. 2). Both SmaI and XhoI restriction digestions allowed differentiation of 31 epidemiologically unrelated strains of *C. botulinum* group II at the strain level, producing 18 and 23 different pulsotypes, respectively. Despite the relatively low number of fragments (5 to 11) produced, the restriction enzyme XhoI was very discriminatory, as evidenced by its high discrimination index (DI) of 0.983 (Table 2).

The analysis of the epidemiologically related strains demonstrated that PFGE was able to group strains from four type E botulism outbreaks into clusters representing four different pulsotypes. PFGE fingerprints of all related food and clinical isolates within each outbreak were indistinguishable using either SmaI or XhoI, indicating that these pulsotypes represent distinct outbreak strains unique to each incident (Fig. 2). Interestingly, the PFGE pattern of SO325E1, a type E stain isolated from shoreline soil of Tasiujaq in 2000, was found to be identical to the PFGE pattern of the FE9508EPB strain isolated from a patient from Tasiujaq who was affected by botulism from eating aged walrus meat (igunaq) in 1995. In

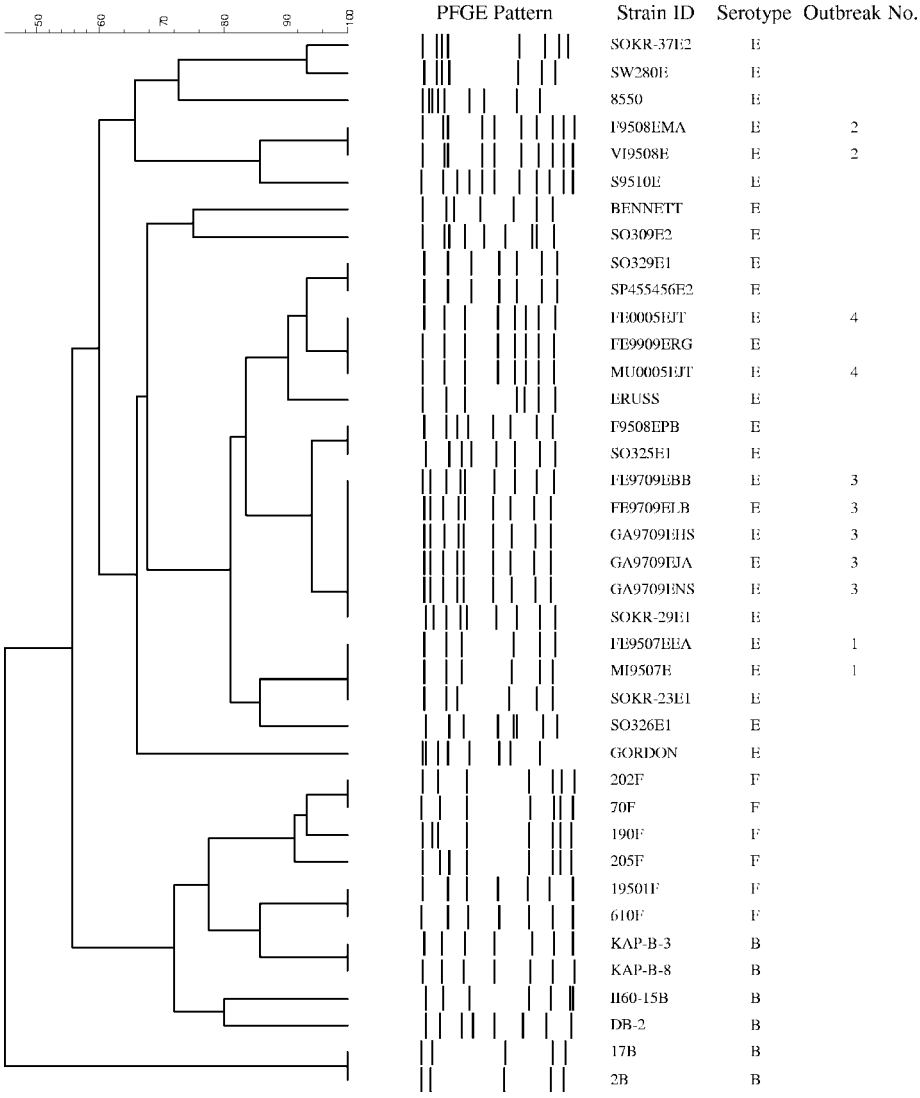

FIG. 2. Dendrogram revealing the high level of genetic relatedness of the 11 epidemiologically related type E strains originating from four different outbreaks analyzed among 39 *C. botulinum* group II isolates and based on their XhoI PFGE patterns. The percentage of similarity among strains was determined using the Dice coefficient, and the clustering was performed by UPGMA.

TABLE 2. Discriminatory ability of PFGE, RAPD, and automated ribotyping for the typing of *C. botulinum* group II strains

Typing method	No. of:			DI[b]
	Strains[a]	Types	Fragments	
PFGE				
SmaI[c]	31	18	7–10	0.951
XhoI	31	23	5–11	0.983
RAPD analysis				
OPJ-13	31	28	3–16	0.994[d]
Ribotyping				
EcoRI	25	2	1–2	0.280

[a] Strains that were epidemiologically related to an outbreak strain were not included.
[b] Numerical index of the discriminatory ability of typing systems (25).
[c] Fragments below 23.1 kb were not included.
[d] The value of the discrimination index is overestimated due to the low reproducibility of the RAPD technique, which had an average coefficient of similarity of 80.5% for duplicate samples with primer OPJ-13.

addition, SOKR-23E1, a type E strain isolated from sediments sampled in a large river that flows into the southern Ungava Bay, had the same PFGE pattern as the food and clinical isolates of outbreak 1 that occurred in Kangiqsualujjuaq, a community located in the southeastern Ungava Bay. PFGE analyses also revealed the high genetic relatedness among isolates originating from a common geographic region or aquatic environment. The PFGE pattern of FE9909ERG, a type E strain isolated from a fecal specimen of a botulism case in Inuvik, Northwest Territories, in 1999 was found to be identical to the PFGE patterns produced by MU0005EJT and FE0005EJT, two epidemiologically related type E isolates recovered during a subsequent outbreak from the same location in 2000. SP455456E2, a type E isolate recovered in 2001 from the surface of a coastal rock used for seal butchering, possessed a PFGE pattern that was indistinguishable from that of isolate SO329E1, a type E strain isolated from a shoreline soil

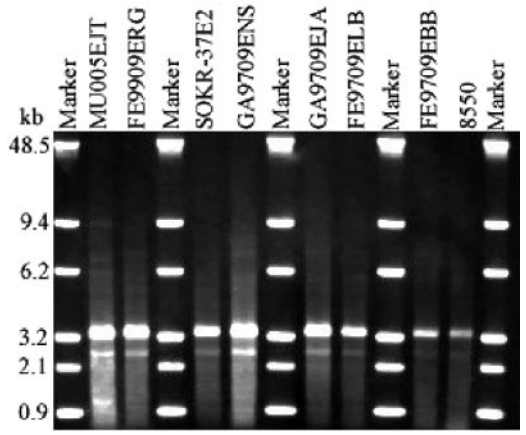

FIG. 3. Typical EcoRI ribopatterns of *C. botulinum* type E strains characterized with only two main lower fragments using an automated RiboPrinter system.

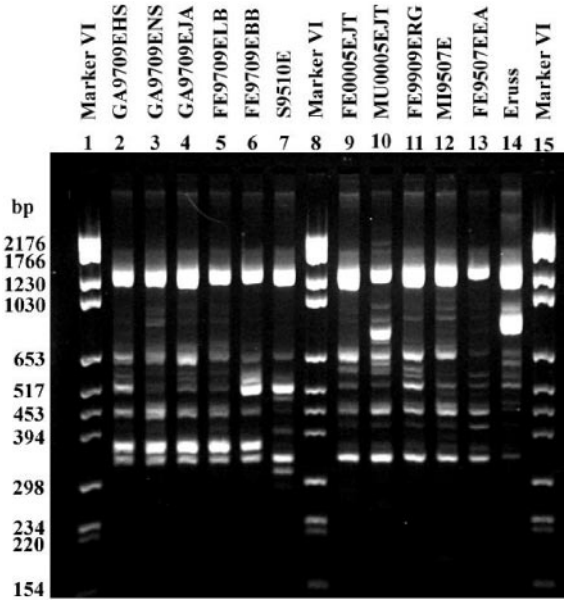

FIG. 4. RAPD patterns of nine epidemiologically related and three epidemiologically unrelated type E strains of *C. botulinum*. Lanes 1, 8, and 15, DNA molecular weight marker VI; lanes 2 to 6, outbreak 3 strains; lanes 9 and 10, outbreak 4 strains; lanes 12 and 13, outbreak 1 strains; lanes 7, 11 and 14, epidemiologically unrelated type E strains.

sample collected near the same coastal rock and at the same time. A type F isolate (19501F) originating from marine sediment, collected at a depth of 1,326 m 100 km off the coast of Oregon in 1965 (17) also had a PFGE pattern identical to that of isolate 610F, which was recovered from a salmon captured in the Columbia River in Oregon State in 1966 (15).

Automated ribotyping analysis. Of the three enzymes tested on group II strains of *C. botulinum*, only EcoRI was able to cleave DNA and generate DNA fragment patterns of good resolution. The EcoRI ribotypes were usually characterized by only two fragments, one intensely stained and one faintly stained, both located in the smaller size range (~3.2 and 2.1 kb, respectively) (Fig. 3). All replicate samples ($n = 7$) included were consistently reproducible. Most PstI and ClaI digestions of 14 and 32 group II strains, respectively, did not produce ribopatterns, regardless of serotype. Many of these ribopatterns were not well resolved, having several faint fragments that could not visually be observed. Of nine PstI (56%) and 16 ClaI (50%) DNA fragment patterns detected by the software of the RiboPrinter, 9 PstI and 14 ClaI ribogroups, respectively, were generated. Thus, the poor typeability obtained with PstI and ClaI and the lack of discrimination (DI = 0.280) resulting from EcoRI digestion made any cluster analysis of ribotyping data meaningless.

RAPD analysis. All of the 39 *C. botulinum* group II strains tested by RAPD generated DNA fragment patterns with both primers used (OPJ-6 and OPJ-13). Both visual and computer analyses showed that the intensity of amplified products of the same strain varied greatly within and between gels. The lack of consistency in fragment intensity and the presence or absence of multiple fragments in one of the duplicate gels caused difficulty in assessing the reproducibility of the procedure.

Pairwise comparison of RAPD duplicates for primer OPJ-6 indicated that the procedure had poor reproducibility for all serotypes of *C. botulinum* group II, with 57.4, 65.4, and 52.3% similarity coefficients for type B, E, and F, respectively. The RAPD fingerprints produced with primer OPJ-6 generally contained a large number of fragments, several of which were minor and very low in intensity (data not shown). The lack of reproducibility was attributed mainly to the presence of non-reproducible faint fragments. However, several occasions, the fingerprints differed because one or two high-intensity fragments were present or absent in duplicate samples. Despite low reproducibility, many OPJ-6 duplicates shared a general, common DNA fragment pattern.

The reproducibility of RAPD patterns performed with primer OPJ-13 was better for all serotypes, with 85.3, 78.5, and 84.8% similarity coefficients for type B, E, and F, respectively. Up to 14 group II strains differed by up to two faint fragments, and regardless of serotype, most duplicate fingerprints shared a general, common DNA fragment pattern. In contrast to the OPJ-6 fingerprints, those generated with primer OPJ-13 were characterized by a lower number of fragments, several of which were large and bright, thereby facilitating the interpretation (Fig. 4).

Cluster analysis was performed with RAPD fingerprints obtained with primer OPJ-13 only. The results of the dendrogram analysis should, however, be viewed with caution due to the lack of reproducibility of the RAPD technique. Each dendrogram consisted of a main cluster of type E strains which diverged from a cluster of type B and F strains (Fig. 5). The RAPD technique was discriminatory, producing up to 28 distinct RAPD types with a DI of 0.994 (Table 2). Among all the epidemiologically related food and clinical type E isolates, only two strains from outbreak 2 had identical RAPD patterns, and five strains from outbreak 3 grouped at a >85% similarity level (Fig. 5). However, the genetic relatedness observed among these strains was not reproduced when they were cultured and RAPD typed at different times. All the other related food and clinical isolates from outbreaks 1 and 4 showed distinct RAPD types. No linkages could be established between the epidemiologically related type E isolates from any of the four outbreaks when only reproducible fragments were considered in

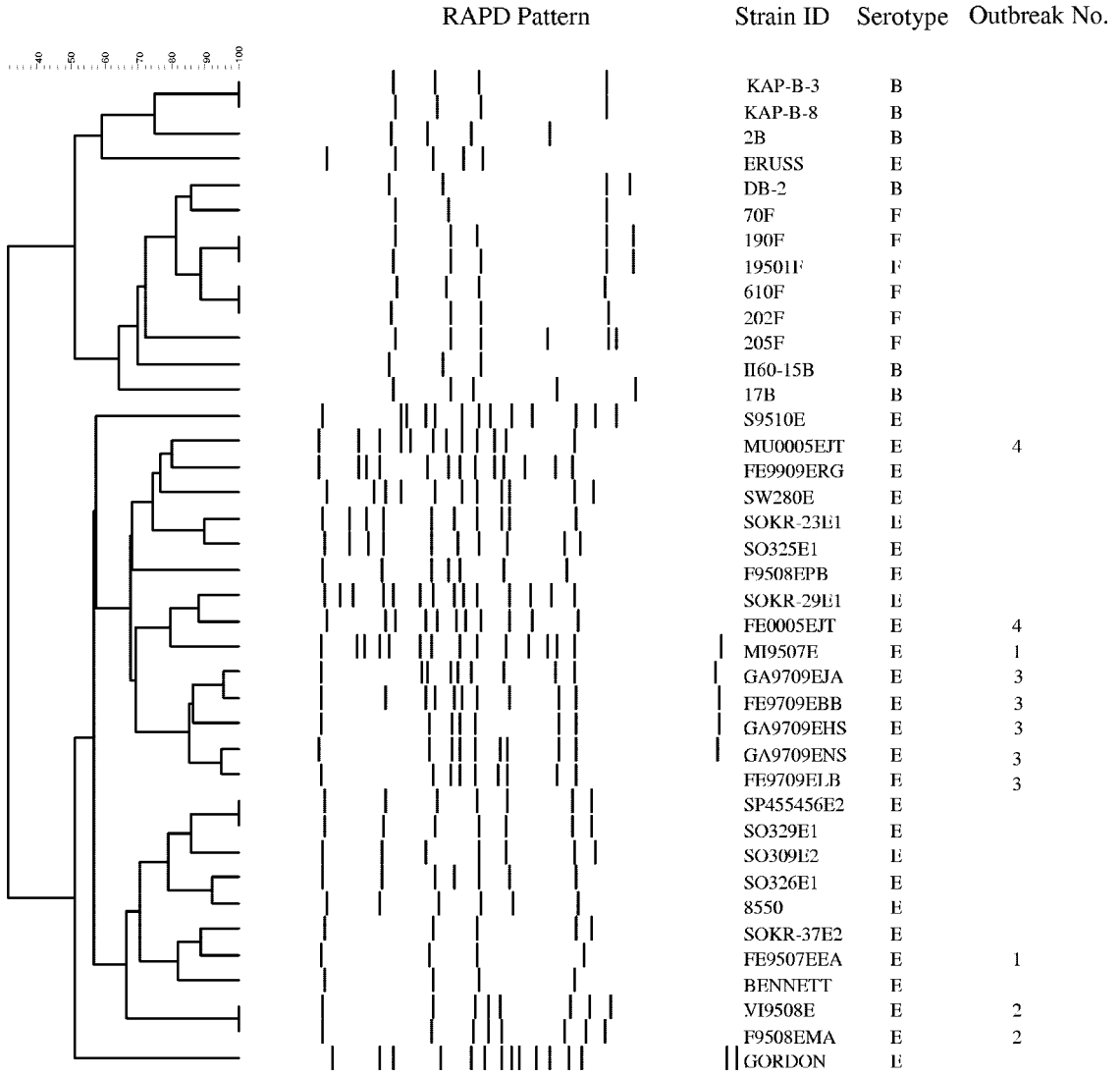

FIG. 5. Dendrogram showing the low genetic relatedness of 9 of the 11 epidemiologically related type E strains originating from four different outbreaks analyzed among 39 *C. botulinum* group II strains and based on their OPJ-13 RAPD patterns. The percentage of similarity among strains was determined using the Dice coefficient, and the clustering was performed by UPGMA.

the analysis, indicating that the RAPD technique artificially discriminated between epidemiologically related strains. However, DNA fragment patterns were reproducibly identical (all fragments included) between KAP-B-3 and KAP-B-8, two type B strains isolated from kapchunka (uneviscerated, salted, air-dried whitefish) in 1981 (12) and between two type E isolates (SP455456E2 and S0329E1) recovered from the same coastal environment.

Comparison of typing methods. With the use of 50 μM thiourea, PFGE had the ability to type all *C. botulinum* group II strains and achieved the same level of typeability as RAPD analysis and automated ribotyping. Both the automated ribotyping and the PFGE procedures were reproducible, as demonstrated with the analysis of replicate samples. In comparison, the duplicate RAPD samples did not consistently produce identical fingerprints from run to run, even with the use of Ready-To-Go RAPD kits, which minimize differences in PCR reagent concentrations. Automated ribotyping using EcoRI had poor discriminatory ability, as most fingerprints displayed only two fragments, which limits its use for epidemiological investigations. PFGE was discriminatory and reproducible, allowing recognition of outbreak strains among unrelated type E strains. While RAPD analysis produced the highest number of different DNA fragment patterns, the technique was not sufficiently reproducible to adequately link the outbreak strains, even when only the reproducible fragments were selected.

DISCUSSION

Food-borne botulism caused by *C. botulinum* type E remains a public health concern in the Canadian Arctic (3–5) and Alaska (46), where the disease occurs sporadically. Molecular characterization of type E outbreak strains has not yet been performed to determine the prevalence and geographical dis-

tribution of different *C. botulinum* subtypes in areas of endemicity. The goal of this study was to optimize and compare PFGE with RAPD analysis and automated ribotyping for use in the molecular epidemiology of *C. botulinum* type E based on performance criteria for typing systems (49).

PFGE is the current gold standard method used to differentiate strains of bacterial pathogens of public health significance, to assess the epidemic spread of infectious diseases in hospitals (32, 48), and to trace food-borne outbreaks (1, 8, 36). Although PFGE is reproducible and discriminatory, some strains of *C. botulinum* types A and E have not been typeable using PFGE (20–22) due to DNA degradation, despite the use of formaldehyde prior to DNA isolation. In this study, however, we have shown that the addition of 50 μM thiourea to the running buffer overcomes the problems of DNA degradation, with all 21 type E isolates that were previously untypeable now producing high-quality fingerprints. At this thiourea concentration, typical smearing was consistently replaced by clear, interpretable DNA macrorestriction patterns that appeared sharp with reduced background. However, both the inactivation of enzymes with formaldehyde prior to DNA isolation and the addition of thiourea during electrophoresis were necessary to achieve the typeability of all *C. botulinum* type E strains tested. Ray et al. (41) first demonstrated that DNA degradation can occur during electrophoresis due to Tris buffer-dependent cleavage of double-stranded DNA, caused by the production of a peracid derivative of Tris formed at the anode (42). The use of thiourea presumably prevents degradation of the DNA by scavenging the nucleolytic Tris derivative (41).

Although it is not known what makes some type E strains sensitive to DNA degradation during electrophoresis while others are resistant, this trait has been associated with specific sites of DNA modifications of *Streptomyces lividans*, which render the DNA susceptible to Tris-dependent cleavage (18). None of the type B and type F *C. botulinum* group II strains in this study showed evidence of DNA degradation without the use of thiourea, indicating that DNA degradation was limited to specific type E strains. Römling and Tümmler (43) occasionally observed that only one isolate from a clonal group of variants of *Pseudomonas aeruginosa* was affected by DNA degradation and suggested that DNA degradation is not a stable trait. Some of the type E strains originally untypeable by PFGE were typed successfully at a different time without the addition of thiourea in the running buffer but only when they were digested with SmaI and not with XhoI. Initial research on the degradation of *S. lividans* DNA during electrophoresis revealed that DNA degradation was dependent upon batches of chemicals used in Tris-acetate-EDTA buffer preparation (53). It was also demonstrated that the addition of ferrous ions to Tris-acetate-EDTA buffer caused the degradation of *S. lividans* DNA (53). Whether the electrophoretic conditions were different between some of the PFGE runs of *C. botulinum* type E DNA due to differences in ferrous iron concentrations in the running buffer or other factors remains unknown, but nonetheless, thiourea was effective, enabling characterization of all type E strains previously not typeable without its inclusion.

PFGE was able to link epidemiologically related isolates from four type E botulism outbreaks using either SmaI or XhoI. Among the 31 epidemiologically unrelated group II strains, 18 and 23 different pulsotypes were produced with SmaI and XhoI, respectively. These results are in agreement with other researchers who found that XhoI was more discriminatory than SmaI for the subtyping of *C. botulinum* type E in fish and fishery products from Finland and other areas (22, 29). However, three epidemiologically unrelated type E isolates could not be distinguished from outbreak strains using PFGE with both restriction enzymes. The PFGE pattern of the sediment isolate SOKR-23E1 originating from the Koksoak River, located approximately 150 km southwest of Kangiqsualujjuaq, was found to be identical to the PFGE patterns of the isolates from outbreak 1, which occurred in Kangiqsualujjuaq. The shoreline soil isolate SO325E1 and the single clinical isolate FE9508EPB, both originating from Tasiujaq, yielded the same SmaI or XhoI pulsotypes but were not epidemiologically related. Also, the epidemiologically unrelated isolate FE9909ERG shared the same PFGE pattern as the isolates from outbreak 4 in Inuvik (isolates FE0005EJT and MU0005EJT). Although these isolates were not epidemiologically related to the outbreak strains, they originated from the same region or northern village where the outbreaks occurred. The genetic biodiversity of *C. botulinum* type E in the Canadian Arctic is unknown, and further studies are needed to determine the distribution of subtypes involved in type E botulism outbreaks associated with the consumption of aged marine mammal meat. All strains belonging to *C. botulinum* group II were typeable by an automated ribotyping system using EcoRI, including the type E strains sensitive to DNA degradation. These results differed from a previous study indicating that DNA degradation was the reason that only 2 of 13 type E strains were typeable with EcoRI (45). The different growth conditions and cell harvesting techniques between the two studies might have resulted in different DNA yields available for restriction digestion and hybridization. However, most EcoRI ribopatterns from *C. botulinum* group II strains were characterized by two fragments of approximately 3.2 and 2.1 kb. These results may indicate that most EcoRI restriction sites are conserved among *C. botulinum* group II and that these strains contained low copy numbers of rRNA operons and/or different rRNA operon copies located on fragments of similar molecular sizes.

This particular EcoRI ribopattern was observed in only three of seven group II strains analyzed by automated ribotyping in another study (45), and several other fragments were resolved in addition to the typical intensively stained fragment of approximately 3.2 kb in 19 group II strains analyzed by manual ribotyping (23). The lack of discrimination by automated ribotyping observed in the present study contrasts with the results of Hielm et al. (23), who found that manual ribotyping was as good as PFGE in differentiating *C. botulinum* group II strains. However, because of difficulties in interpreting fragments in the region above 3 kb, the authors recognized that manual EcoRI ribotyping was not optimal for group II strains. The presence of some fragments larger than 3 kb was observed in some ribopatterns generated in the present study, but these were too weak in intensity to be selected as true fragments. A comparison of the two ribotyping techniques for the differentiation of clinical isolates of *Vibrio cholerae* O1 ribotypes also showed that small fragments weak in intensity that were detected in ribopatterns generated by manual ribotyping were not observed by the automated system (16).

Automated ribotyping did not allow the differentiation of *C. botulinum* group II strains below the group level when EcoRI

was used. A previous study (45) of the analysis of *C. botulinum* groups I and II by automated ribotyping using EcoRI showed that type B strains from group II can cluster under the same ribogroup as type B strains from group I, despite the evidence that these groups belong to two evolutionarily distinct lineages, based on 16S rRNA gene sequences (13). These unexpected findings have raised questions about the identities of some group II strains that were analyzed by automated ribotyping and were addressed in another report (24). In that study, all EcoRI ribopatterns of group II isolates were similar to each other but very different from group I ribopatterns observed in our laboratory (data not shown). Our current database of ribotypes of *C. botulinum* group I and group II supports a clear taxonomic divergence between these two groups, in good agreement with the results obtained by manual ribotyping (24). Based on 16S rRNA sequence data, strains of nonproteolytic *C. botulinum* types B, E, and F form a single phylogenetic group (27). The level of discrimination achieved by PFGE and RAPD analysis was higher than automated ribotyping. Dendrograms generated from both RAPD and PFGE results indicated one large cluster of type E strains separated from another cluster composed of type B and F isolates. Cluster analysis of EcoRI ribopatterns of *C. botulinum* group II produced by manual ribotyping also showed clustering of type B and F strains separate from type E strains (23).

Although all *C. botulinum* group II strains could be typed using RAPD analysis, the technique was hampered by its low reproducibility, particularly with primer OPJ-6. Even with an average coefficient of similarity of 80.5%, primer OPJ-13 was not capable of adequately grouping most epidemiologically related isolates. Our results contrast with those of Hyytiä et al. (28), who obtained highly reproducible results with RAPD analysis of *C. botulinum* groups I and II using the OPJ-6 and OPJ-13 primers. However, they did not specify if all faint fragments, including those that were not reproducible, were included in their analysis of reproducibility. In our study, all fragments were included in the analysis, which means that two replicate samples differing by only one faint fragment were considered two different RAPD patterns. Several factors affecting the reproducibility of arbitrary PCR-amplified methods have been well documented (37, 51) and include the selection of primers (50). Bidet et al. (9), working with *C. difficile*, obtained a reproducibility of 100% for PCR ribotyping and PFGE analysis, while the reproducibility for arbitrarily primed PCR ranged from 33 to 88%, using three different 10-mer primers.

Although the RAPD method using OPJ-13 gave the highest DI among the three typing methods, this result should be interpreted with caution as the reproducibility of the method was not 100%, which is usually required to calculate the DI based on Simpson's diversity index. The effect of reproducibility on the discriminatory indices of typing systems has been previously shown by Hunter (26), who proposed the use of a standardized DI based on a predetermined reproducibility for adequate comparison. However, this method of standardizing the DI was not tested using typing methods that generate DNA fragment patterns. Such an application would be difficult when the typing methods under comparison yield remarkably different numbers of fragments, such as RAPD analysis and automated ribotyping.

Fingerprinting analysis of RAPD patterns of *C. botulinum* type E has been traditionally performed by visual examination, presumably because of the presence of a large number of low-molecular-weight fragments and the frequent occurrence of faint fragments, which make computer analysis more difficult (29). To control variation in DNA fragment patterns from run to run, nonreproducible, faint fragments were excluded from the analysis of RAPD patterns (29). Such fingerprint analysis is time-consuming and would be very challenging when large datasets are being analyzed. The epidemiological concordance of RAPD analysis performed with OPJ-13 did not improve among any of the epidemiologically related strains of the four outbreaks by selecting only reproducible, faint fragments.

A comparison of typing methods showed that an optimized PFGE incorporating thiourea was the only fingerprinting method able to successfully identify epidemiologically related strains of *C. botulinum* type E belonging to four different outbreaks. The RAPD technique was found to be unreliable for the molecular typing of group II strains and is therefore not recommended for epidemiological investigations. Despite being reproducible, an EcoRI automated ribotyping system did not allow differentiation of *C. botulinum* group II strains below the group level. A new set of restriction enzymes may improve the discriminatory power of the automated ribotyping system. Our results have shown that PFGE is a reliable and discriminatory molecular typing method for the investigation of outbreaks of type E botulism and molecular epidemiological studies in the Canadian Arctic.

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