Automated Ribotyping for the Identification and Characterization of Foodborne Clostridia

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MS 06-089: Received 16 February 2006/Accepted 3 July 2006

ABSTRACT

The DuPont Qualicon RiboPrinter was employed to determine if automated ribotyping could be used to differentiate between and characterize various species of foodborne clostridia. EcoRI digests were used to ribotype 49 isolates that represented seven Clostridium species: C. aerotolerans, C. beijerinckii, C. botulinum, C. butyricum, C. perfringens, C. putrificum, and C. sporogenes. EcoRV digests were also used to ribotype 17 C. botulinum isolates to determine if an alternate restriction enzyme was more suitable than was EcoRI for toxin typing. It was concluded that the RiboPrinter could be potentially used to identify most of the clostridia represented in the study, but that the system has difficulty distinguishing between C. botulinum and C. sporogenes. The system may also be potentially used to characterize clostridia based on phenotypic characteristics. Toxin typing of clostridia remains problematic, but may be improved by the use of restriction enzyme combinations.

Members of the genus Clostridium are predominantly gram-positive, anaerobic, spore-forming rod-shaped bacteria. Most species are obligate anaerobes, and are taxonomically distinguished by the proteolytic activity of some members of the genus. C. botulinum produces toxins and is a threat to human health. There are an estimated 449 cases yearly of botulism worldwide (5), with a fatality rate as high as 65% (9). Although C. butyricum has been chiefly associated with acidic food spoilage in tomatoes and other fruits, this organism was determined to be the cause of a botulinal-type disease in two young children in Italy (12). Preformed toxin in food from C. perfringens can cause illness. The bacterium can also colonize the gut and cause intermembranous colitis. There also was a link postulated between C. perfringens intoxication and some cases of sudden infant death syndrome (11). C. botulinum, C. sporogenes, C. perfringens, and C. putrificum have caused spoilage in canned and other foods, especially when food was cooled too slowly after cooking. Other clostridia such as C. beijerinckii and C. aerotolerans have been isolated from food and are suspected of being the causative agent in rare cases of food discoloration.

Rapid methods of identification and characterization of species of the genus are of a great benefit to scientists that conduct epidemiological investigations. Several techniques have been developed to identify and characterize Clostridium spp. and clostridial toxins. The mouse bioassay is used as the standard method for the detection of C. botulinum toxin (16). Its presence is indicated by typical symptoms of botulimum intoxication and death after intraperitoneal injection. Enzyme-linked immunosorbent assays (ELISAs) have also been developed that detect and distinguish between the various botulinal neurotoxins (13, 17). These ELISAs were found to be more sensitive than was the mouse bioassay.

Nucleic acid–based methods may hold the promise of being the most reliable and reproducible for identifying and characterizing clostridia, as they do not rely on phenotypic characteristics such as is the case for biochemical or immunological diagnostic tests. Nucleic acid–based methods for characterization are often based on random fragment length polymorphism of restriction endonuclease digests of conserved regions of DNA such as the ribosomal rRNA operon. Southern blotting can be used to visualize the polymorphisms of the rRNA operon in a technique called ribotyping. This method, sometimes in concert with PCR, has been used for both rapid identification and characterization of C. difficile (1, 4, 14). DuPont Qualicon (Wilmington, Del.) developed the RiboPrinter, which is an automated robotic platform that performs all the steps necessary for ribotyping of bacteria by the Southern blot technique. Statistical algorithms are used to compare the digitized image of the resulting band patterns to a library of bacterial ribotype patterns that are based on restriction digests of bacterial DNA by EcoRI, PvuII, and PstI. The operation of the instrument is described in detail by Bruce (2).

The RiboPrinter was employed in this study to determine if this method of ribotyping using an automated system is suitable to speciate various clostridia, and to characterize among isolates of the same species in terms of metabolite production. Forty-nine isolates that represented seven Clostridium species (29 C. botulinum, 3 C. putrificum, 4 C. perfringens, 6 C. sporogenes, 3 C. beijerinckii, 2 toxigenic C. butyricum, and 2 C. aerotolerans) were ribotyped based on EcoRI digests. Seventeen C. botulinum isolates were ribotyped using EcoRV digests to determine if other restriction enzymes are as effective as is EcoRI,
which is the primary restriction enzyme on which the largest RiboPrinter identification library is based.

MATERIALS AND METHODS

Bacterial isolates. The cultures that were used in this study came from several sources. The proteolytic C. botulinum strains that were used were obtained from the U.S. Food and Drug Administration (Washington, D.C.). Nonproteolytic C. botulinum strains were provided by the National Marine Fisheries Service (Seattle, Wash.). The two strains that were designated as putrefactive anaerobes (PA), as well as two toxigenic C. butyricum strains were provided by the Food Products Association (Washington, D.C.). The remaining Clostridium spp. were obtained from the American Type Culture Collection (Manassas, Va.) or from culture collections at Kraft Foods (Glenview, Ill.).

Preparation of cell suspensions. Cell suspensions for C. botulinum, C. sporogenes, and other putrefactive clostridia were prepared as described by Skinner et al. (15). An egg yolk agar was inoculated with 1 ml of either a pure spore or crude cell and spore suspension that had a concentration of 10^6 to 10^9 CFU/ml. The egg yolk agar plates were then incubated anaerobically for 24 to 48 h at 35°C. The surface of the agar was gently rubbed with a sterile inoculating loop as the plate was washed with 1 to 3 ml of sample buffer (2 mM Tris, 20 mM EDTA, pH 8.0). All cell suspensions from other Clostridium spp. were prepared from pure spore suspensions or pure crude cell and spore suspensions after inoculation onto brain heart infusion agar (Difco, Becton Dickinson, Sparks, Md.), in the same manner as with the C. botulinum strains.

Automated ribotyping. From each cell suspension 30 μl was placed into a separate tube of the sample carrier, which was then placed into a heating block at 80°C for 10 min. After cooling, 5 μl of lysostaphin and 5 μl of N-acetylmuramidase lysing agents were added to each tube of the carrier. For C. butyricum and nonproteolytic C. botulinum, 3 μl of formaldehyde (37%) was also added, and the cell suspensions were placed in an ice bath for 1 h prior to being placed into the heating block. After sample carriers were loaded into the instrument, the automated process was initiated. The automated platform contained DNA preparation, separation and transfer, membrane processing, and detection modules, with transfers performed by pipettes mounted on robotic arms. The sample carrier was loaded into the DNA preparation module along with the DNA preparation pack that holds the ethidium bromide dye, marker DNA, lytic enzymes, and a place for a vial of the restriction endonuclease of choice. In this study either EcoRI (DuPont Qualicon) or EcoRV (Promega, Madison, Wis.) was used.

In the DNA preparation module the cells were lysed, and the released DNA was restricted. The sample and DNA markers were then transferred to the agarose gel cassette in the separation and transfer module. After electrophoresis the membrane was moved against the gel, which allowed restriction fragments to be transferred to the membrane and immobilized. The membrane was then moved from the separation and transfer to the membrane processing module, where denatured membrane-bound DNA was hybridized with a labeled probe for the rRNA operon and treated with a DNA antibody–alkaline phosphatase conjugate. A series of wash steps were then performed to remove unbound conjugate on the membrane, after which a chemiluminescent substrate was applied.

The membrane was then moved to a charged-coupled device camera in the detection module that detected luminescing DNA fragments and converted the information from the patterns into a digitized image, which was then stored on the computer hard drive. System software distinguished between sample and marker lanes, compared the intensity of marker fragments with those of the sample fragments, and normalized the data that were extracted from the image. A proprietary algorithm converted this digital information into a densitometric representation that was the riboprint—a lane of light and dark bands that reflects the molecular weight and band intensity of the labeled DNA fragments. Software then used these parameters of the digitized image to compare sample patterns with those of two different riboprint pattern libraries, and generated similarity values of the sample strains to those of the libraries (a value of 1.00 is a perfect match). In one library, riboprints produced in the work presented here were grouped together based on a similarity value of approximately 0.93 or higher. Such ribogroups were then stored as a digital representation of the average of all isolate riboprints that had similarity values above this threshold.

The system also compared each new riboprint with the second, internal library of fingerprints provided by DuPont Qualicon. Identification (assignment of a species name for the organism) was made when isolate riboprints were matched with the other library patterns of this library and a similarity value of approximately 0.85 or greater was achieved when compared with the closest library match. These identification library riboprint patterns were generated from organisms that had been identified with other methods in a validated protocol that was conducted by the manufacturer. A comprehensive description of these automated steps can be found in Bruce (2).

RESULTS

Twenty-six ribogroups were generated from the 49 Clostridium spp. isolates that were ribotyped by the RiboPrinter with EcoRI digests. The digitized fingerprint patterns that were generated by the instrument software for the clostridia that were digested with EcoRI are shown in Figure 1. The riboprints shown here were sorted based on riboprint pattern similarity. There were nine ribotype groups that were generated from the 29 C. botulinum isolates from EcoRI digests, with 22 (75.9%) of the strains being placed into three ribogroups.

The largest of these ribogroups was 1691-4, which contained 10 isolates (34.5% of all C. botulinum isolates). The ribogroup contained patterns from seven C. botulinum neurotoxin type A (BoNT/A) producers and one C. botulinum neurotoxin type B (BoNT/B) producer, as well as two C. sporogenes isolates (PA 72-142 and PA 6982). The next largest C. botulinum ribogroups contained six isolates each. Ribogroup 1689-7 had only BoNT/B producers. Ribogroup 1689-4 had five BoNT/A and one BoNT/B producer. Fourteen C. botulinum isolates were identified by the system as C. sporogenes (13 matches to DUP-18259 and 1 to DUP-14704). These isolate riboprints were found only in ribogroups 1691-4 and 1689-7, which when compared with one another have a similarity value of 0.92 (Fig. 2). The other C. botulinum ribogroups contained three or fewer isolates in each group.

On two attempts to ribotype C. botulinum 53-B, the riboprints were placed into two different ribogroups (1689-4 and 1692-3) that also contained other C. botulinum isolates (Fig. 1). The riboprint of another C. botulinum iso-

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FIGURE 1. Clostridia ribotyped with EcoRI digests. All C. botulinum isolates are group I; proteolytic strains with the toxin type represented in the name of each strain. Shown are full ribogroup designations as well as the digitized riboprint pattern of each isolate. The patterns are listed by riboprint similarity with members of each successive ribogroup alternately shaded and unshaded. Riboprints of isolates that were placed into the same ribogroup after repeated attempts are not shown.

FIGURE 2. EcoRI ribogroups of C. botulinum with similarity values between adjacent ribogroups. Ribogroups are listed by riboprint pattern similarity, and are an average of all isolate riboprints placed into a single group.

late, Gar51-A, was also placed into two different ribogroups. One ribogroup (1691-1) also contained two other C. botulinum isolate riboprints. The other ribogroup into which Gar51-A was placed (1688-1) also contained the riboprint from the isolate Clostridium sp. 05/98-1 that is believed to be C. putrificum.

There were five ribogroups that were generated for six C. sporogenes isolates. As stated previously the two C. spo-
of Clostridium for automated ribotyping presented problems. Some of the difficulties in obtaining adequate cell suspensions were thought to be because of the application of the cell suspension preparation protocol recommended by the manufacturer to organisms that sometimes produce extracellular material or develop spores. Bacteria such as Bacillus and Clostridium produce exopolysaccharides that make it difficult to obtain enough biomass and ultimately, enough genetic material for analysis. For cell suspension preparation the system protocol specified that the tip of a small, cylindrically-shaped, sterile stick be pressed onto a cultured lawn of bacterial culture perpendicular to the plane of the surface of the agar. This method may not allow enough cells to be transferred easily among capsular material. The method of Skinner et al. (15) was used to prepare the cell suspensions for this study. Here the cell lawns were flooded with sample buffer (2 mM Tris, 20 mM EDTA), and sloughed off with a sterile loop, which created turbid cell suspensions. This increased the probability that enough vegetative cells were harvested and available for lysis, even if some capsular material was present. Culture lawns were not allowed to grow more than 48 h to minimize spore development, as spores have a hard, calcium-containing outer coat that resists cell lysis.

It was also difficult to ribotype DNA from group II C. botulinum isolates. This problem was thought to be because of the inability in many cases to obtain nondegraded DNA, as was reported by Heilm et al. (7) in a study that used pulse-field gel electrophoresis for genomic analysis of group II C. botulinum, which has caused more outbreaks

DISCUSSION

The preparation of cultures of *Clostridium* for automated ribotyping presented problems. Some of the difficulties in obtaining adequate cell suspensions were thought to be because of the application of the cell suspension preparation protocol recommended by the manufacturer to organisms that sometimes produce extracellular material or develop spores. Bacteria such as *Bacillus* and *Clostridium* produce exopolysaccharides that make it difficult to obtain enough biomass and ultimately, enough genetic material for analysis. For cell suspension preparation the system protocol specified that the tip of a small, cylindrically-shaped, sterile stick be pressed onto a cultured lawn of bacterial culture perpendicular to the plane of the surface of the agar. This method may not allow enough cells to be transferred easily among capsular material. The method of Skinner et al. (15) was used to prepare the cell suspensions for this study. Here the cell lawns were flooded with sample buffer (2 mM Tris, 20 mM EDTA), and sloughed off with a sterile loop, which created turbid cell suspensions. This increased the probability that enough vegetative cells were harvested and available for lysis, even if some capsular material was present. Culture lawns were not allowed to grow more than 48 h to minimize spore development, as spores have a hard, calcium-containing outer coat that resists cell lysis.

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of human botulism than has any other (6). Three causes have contributed to the inability to type this group: low yields of genetic material, inadequate cell lysis, and the action of extracellular DNases (7).

Treatment of the initial cell suspension with formaldehyde allowed Hielm et al. (8) to successfully type group II C. botulinum with pulse-field gel electrophoresis as well as ribotyping. The use of formaldehyde was thought to inhibit the activity of extracellular DNases. Such a formaldehyde treatment was incorporated into the cell suspension preparation steps prior to ribotyping in this study. In this way strains 17-B and D8-E were ribotyped. Subsequent attempts to ribotype group II strains were unsuccessful in spite of the addition of formaldehyde along with a heat treatment of cell suspensions at 90°C for 1 h.

Automated ribotyping would prove to be a benefit for the characterization of isolates of C. botulinum if random fragment length polymorphism fingerprints obtained with ribotyping could be used as a basis to predict proteolytic activity or type of toxin production. The proteolytic and nonproteolytic strains of C. botulinum were found in previous studies to be phylogenetically divergent by Collins and East (3), which confirmed that proteolytic activity may be used as part of the grouping of strains of C. botulinum. Skinner et al. (15) employed the RiboPrinter to ribotype C. botulinum, using EcoRI digests, and found that group II strain 17-B was placed into the same ribogroup as group I proteolytic strains 642-B and 383-B. It was concluded that the system may not be useful for taxonomic studies. Using an EcoRV digest in this study, it was found that 17-B was not placed into a ribogroup that also included group I 642-B and 383-B isolates. The use of additional restriction enzymes other than EcoRI might allow the characterization of isolates of group I and II C. botulinum, based on automated ribotyping as was found with manual ribotyping using Clal, EcoRI, EcoRV, HindIII, and SpeI (8).

There was some agreement here with the constituent strains of the ribogroups of C. botulinum obtained by Skinner et al. (15) in a study that also featured automated ribotyping to differentiate types and strains of C. botulinum by EcoRI digests. Instruments in both labs placed Clovis-A and 4896-A into the same ribogroup. Strain OS3-A was also placed into this group by Skinner and his colleagues, while OS2-A was placed into this group by riboprinting in this lab. Strain 4896-A was isolated from a stool sample of an individual that contracted botulism. OS2-A and OS3-A were isolated from onion skins implicated in the same outbreak. Strains 383-B and 642-B, which were isolated from canned mushrooms between 1972 and 1973, and Mushroom 2-B, which was isolated from fresh mushrooms in 1975, were also placed into the same ribogroup at both labs. There was also some disagreement. Strain 73-A was not placed into the same group as 62-A as in this study. Additionally 169-B did not fall into the same ribogroup as Clovis-A, PS2-A, and 4896-A, as in the study by Skinner et al. (15).

It was interesting that the largest EcoRI-based ribogroup in this study (1691-4), which was composed of predominantly BoNT/A producers, had a similarity value of 0.92 as compared with a ribogroup that had only BoNT/B producers. It would therefore appear that automated ribotyping might not provide the basis to characterize C. botulinum for the production of BoNT/A and BoNT/B. Alternatively, seven of the isolates in EcoRI-based 1691-4 also occurred in the largest EcoRV-based ribogroup, 2098-1, which contained only BoNT/A producers. The use of a digest that is created with both EcoRI and EcoRV or combinations of other restriction enzymes might improve the chances for characterization. Use of restriction enzymes, such as RsaI, that recognize four base pair–long sites would allow more potential restriction sites to be sampled for each DNA preparation, and thus has the potential to aid in discrimination. This advantage may be offset, however, by the large number of fragments produced in these instances, which may lead to problems such as nonhomologous fragments that appear to be identical because of comigration.

Aside from the two C. sporogenes isolates (PA 72-142 and PA 6982) that were placed into the largest C. botulinum ribogroup, all C. sporogenes were placed into separate ribogroups. Characterization of this species in terms of spore development and heat resistance might be accomplished by automated ribotyping. All C. sporogenes were correctly identified by the system; however, 14 of the C. botulinum strains and all nine isolates placed into ribogroup 1691-4 were identified as C. sporogenes. It may be that the system can distinguish between C. sporogenes and C. botulinum when attempting to identify C. sporogenes, but cannot distinguish between C. sporogenes and C. botulinum when identification is attempted for C. botulinum.

The four C. perfringens isolates ribotyped in this study were placed into different ribogroups. In another study in which 111 C. perfringens isolates obtained from industrially produced ground meat were ribotyped manually, 107 distinct ribotype groups were produced (10). This not only automated ribotyping potentially useful for strain tracking of C. perfringens, but perhaps a potential tool to characterize this organism in terms of phenotypic traits such as production or specificity of toxin.

Some similarity was seen between portions of the riboprint patterns of the three C. beijerinckii and two toxigenic C. butyricum (5520-E and 5262-E) isolates. These are also adjacent patterns in the listing of clostridia by similarity in Figure 1. One of the C. beijerinckii strains (ATCC 8260) was originally deposited with the identification of C. butyricum. Both species produce butyric acid, which may explain why C. beijerinckii has often been cited in the literature as C. butyricum. An analysis of random fragment length polymorphism patterns for these species compared with other clostridia may allow for a simple, qualitative characterization of riboprints based on the production of butyric acid. The C. butyricum isolate riboprints of 5520-E and 5262-E, in spite of their ability to produce a botulinum-type neurotoxin, also were distinctly different from patterns obtained from the other C. botulinum. This finding supports the hypothesis that the presence of the gene that encodes BoNT/E in these C. butyricum strains was determined to be the causative agent of a botulinum-type intoxication of two children in Italy in the mid-1980s was
likely because of horizontal gene transfer from *C. botulinum* (12).

It was found in this study that ribotyping with the automated RiboPrinter may prove suitable for characterization of some clostridia, but restriction enzymes other than that which is used in the standard system protocol, EcoRI, may be needed. The system has also shown some promise in the identification of foodborne clostridia, although some species such as *C. sporogenes* and *C. botulinum* that are closely related phylogenetically may prove difficult to definitively identify without supplementary testing. It was also necessary to modify the cell suspension preparation protocol to add more time (up to an additional 24 h) for enough biomass to grow. Thus a wash of the entire plate was necessary to obtain enough genetic material for ribotyping. Although other typing methods such as pulse-field gel electrophoresis may be more discriminatory, automated ribotyping is much less labor-intensive, diminishes the need for highly skilled technicians, promotes the reproducibility of results, and performs the statistical analysis for pattern comparison that is also archived for the comparison of bacteria isolated in the future. The current instrument library for clostridia is small, but personal libraries (custom identification libraries) can be constructed so that isolates that are confirmed as particular *Clostridium* spp. can be used for subsequent identification and characterization.

ACKNOWLEDGMENTS

We thank Dr. Guy Skinner (U.S. Food and Drug Administration) and Dr. Sebastian Heilm (Department of Food and Environmental Hygiene, Helsinki University, Helsinki, Finland) for personal communication and for sharing insights in regard to the preparation and treatment of cell suspensions.

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