Genotyping *Clostridium botulinum* toxino-type A isolates from patients using amplified rDNA restriction analysis

M. Pourshafie, P. Vahdani and M. Popoff

1 Pasteur Institute of Iran, Department of Bacteriology, Pasteur Avenue, Tehran, Iran
2 Logman Hospital, Department of Infectious Diseases, Tehran, Iran
3 Institut Pasteur, Unité des Anaérobies, 25–28 Rue du Dr Roux, F-75724, Paris Cedex 15, France

In this study, the application of amplified rDNA restriction analysis (ARDRA) for characterizing *Clostridium botulinum* toxino-type A strains isolated from individuals with botulism was evaluated. Ten restriction enzymes were tested for their suitability in ARDRA as a typing method and *Hha*I was selected for the best outcome. Analysis of *Hha*I restriction profiles of the amplified products divided *C. botulinum* isolates into three clusters. Non-toxigenic *Clostridium sporogenes* strains showed an ARDRA restriction pattern that was distinct from those observed for *C. botulinum*. The successful use of ARDRA for sub-division of *C. botulinum* in this study confirmed that this technique is a powerful method for typing of *C. botulinum* toxino-type A clonal diversity. In addition, it is rapid, sensitive and simple.

**INTRODUCTION**

The toxin released by *Clostridium botulinum* acts as a neuromuscular blocking agent, which causes paralysis (botulism) by preventing the release of neurotransmitter from motor neurons (Simpson, 2004). Globally, around 450 outbreaks of *C. botulinum* are recorded annually, of which 90% of cases are caused by home-prepared or preserved foods (Hatheway, 1995). Previously, we reported the first documented outbreak of botulism following consumption of cheese contaminated with *C. botulinum* type A in Iran (Pourshafie et al., 1998).

The standard diagnosis for botulism has been based on detection of the botulinum toxin in clinical and food samples. The toxin is divided into seven toxino-types (A–G) according to their antigenic properties. The toxin is produced by strains belonging to different *C. botulinum* groups (groups I–III) or different *Clostridium* species such as *Clostridium butyricum*, *Clostridium baratii* and *Clostridium argentinense*. Therefore, it has been suggested that reconsid-eration be given to the taxonomy of neurotoxigenic *Clostridium* strains, which is currently based on traditional detection methods (Collins & East, 1998), and that DNA fingerprinting should be used as a supplement to the botulinum neurotoxin production assay and phenotypic characteristics (Hyytiä et al., 1999a).

There is a lack of extensive information about the genetic biodiversity of the food-borne *C. botulinum* strains and the taxonomy of the species is under reconsideration (Hyytiä et al., 1999b). Recent reports have analysed with certain success the genomic diversity of *C. botulinum* strains using PFGE (Nevas et al., 2005), randomly amplified polymorphic DNA (RAPD) analysis (Franciosa et al., 2004) and ribotyping (Skinner et al., 2000). PFGE has been suggested to be suitable for subtyping of the *C. botulinum* group II. The major limitation of PFGE, however, is the requirement for technical skills, equipment and the long duration of the typing protocols. Since molecular typing is the key to more accurate and reliable investigation of botulism outbreaks, the new genotyping method should be reproducible and easy to use.

Amplified rDNA restriction analysis (ARDRA) amplifies specific regions of the 16S and 23S rRNA genes followed by restriction digestion of the amplicon, which, in turn, provides valuable taxonomic information on the isolates. ARDRA has been described for identification of a number of species including *Lactobacillus* (Moreira et al., 2005), *Streptococcus* (Sasaki et al., 2004) and *Mycobacterium* (Kurabachew et al., 2003).

The objective of the present study was to evaluate the applicability of ARDRA for differentiation of *C. botulinum* toxino-type A strains isolated from patients with botulism in Iran.

**METHODS**

**Bacterial growth.** Twelve *C. botulinum* toxino-type A strains and three environmental strains of *Clostridium sporogenes* were studied (Table 1).
C. botulinum strains were isolated from faeces of infected individuals. Six strains were isolated from an outbreak in Iran, four were collected from sporadic cases of infected people in Iran and one strain was isolated from a patient with botulism in France. Strain C. botulinum ATCC 25763\textsuperscript{T} and three strains of \textit{C. sporogenes} were also included. Cultures were grown on anaerobic egg yolk agar from which the colonies were isolated and grown for 3 days on tryptcase–peptone–glucose–yeast extract broth. Cultures were incubated at 37°C in an anaerobic jar with an internal atmosphere of 85% N\textsubscript{2}, 10% CO\textsubscript{2} and 5% H\textsubscript{2}. For biochemical analysis of the strains, Rapid ID 32 A (bioMérieux) was used.

Identification of \textit{C. botulinum} toxinoType A strains. Each strain was identified by standard methods including carbohydrate fermentation, GC of fermentative end products, scoring the presence of the neurotoxin gene with PCR as described elsewhere (Franciosa \textit{et al.}, 1997) and by typing the toxin in the culture supernatants using the mouse toxicity assay. DNA was isolated using the InstaGene purification matrix (Bio-Rad). Extracted DNA was dissolved in 100 μl TE buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA). Purified DNA was aliquoted and stored at –20°C.

\textbf{PCR amplification for ARDRA.} Briefly, each 70 μl mixture contained 0-2 mM each dNTP, 0-4 pmol each primer, 7-5 μl reaction buffer, 1-1 mM MgCl\textsubscript{2}, 0-05% W-1 detergent, 2-5 U Taq polymerase (all from Gibco-BRL Life Technologies) and 42 μl double-distilled water. The sequences of the primers were 5’-AGAGTTTGATC(C/T)GGCTCAG-3’ (5’ end of the 16S rRNA gene) and 5’-GGGATTCAGAAGTGGCCAAA-3’ (5’ end of the 23S rRNA gene). Amplification was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer). After initial denaturation at 94°C for 5 min, the reaction mixture went through 25 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 3 min, followed by a 7 min extension period at 72°C. The presence of PCR products was checked by agarose (0-8%, w/v)/ethidium bromide gel electrophoresis. A negative control (no template DNA added) and strain ATCC 25763\textsuperscript{T} (positive control) were included in each PCR run. Duplicate PCRs were routinely carried out to verify the reproducibility of fingerprints. The length of the amplicon was approximately 4.5 kbp.

\textbf{Enzymic digestion.} Enzymic digestion was carried out for 2 h at 37°C in 17 μl of the amplified DNA and 3 μl of each of the commercially supplied buffer with \textit{HhaI}, \textit{Alul}, \textit{Mbol}, \textit{MspI}, \textit{RsaI}, \textit{XbaI}, \textit{Sau3AI}, \textit{MluI}, \textit{KpnI} or \textit{XhoI} restriction enzyme added. Enzymes were purchased from Gibco-BRL. The restriction fragments were analysed by gel electrophoresis in 3% (w/v) Metaphor agarose (FMC Bioproducts) in electrophoresis buffer (89 mM Tris/HCl, pH 8.0; 89 mM boric acid; 2 mM EDTA) for 5 h at 160 V.

\section*{RESULTS}

All 11 strains isolated from infected patients were identified as \textit{C. botulinum} toxinoType A by PCR and mouse toxicity assay. The \textit{C. botulinum} strains showed identical phenotypes such as carbohydrate fermentations and culture characteristics (data not shown). The pattern of fermentation end products as analysed by GC was similar for the strains studied here.

The 16S and 23S rRNA genes of each of the strains, including strain ATCC 25763\textsuperscript{T} and three strains of \textit{C. sporogenes}, were amplified and then digested with 10 restriction enzymes. There was no enzymic digestion when the extracted DNA was treated with \textit{MluI}, \textit{XbaI}, \textit{MspI} or \textit{Sau3AI} restriction enzymes. Several other enzymes – \textit{Mbol}, \textit{RsaI} \textit{KpnI}, \textit{Alul} and \textit{XhoI} – generated two to four fragments that did not discriminate the strains studied here (data not shown).

Of the enzymes tested for typing of \textit{C. botulinum} group A, only \textit{HhaI} produced recognizable bands that enabled differentiation amongst the isolates. Fig. 1 shows the ARDRA patterns of strains digested with \textit{HhaI}. The number of fragments generated by digestion of \textit{C. botulinum} toxinoType A genomes with \textit{HhaI} ranged from six to nine. The smallest and largest fragments were about 150 and 1200 bp, respectively.

Overall, three restriction patterns with \textit{HhaI} were observed following digestion of the rRNA genes of the 12 \textit{C. botulinum} toxinoType A strains. The strains in lanes 1, 2, 4, 9, 10 and 11 (Fig. 1) were isolated from a outbreak in Iran and were considered to be pattern 1, with nine fragments corresponding to molecular sizes of 1-20, 1-10, 0-87, 0-75, 0-50, 0-40, 0-35 and 0-25 kbp. An additional fragment of 0-15 kbp was present in some of the isolates of pattern 1. The band of 1-2 kbp was common to all of the strains studied here. Pattern 2 was observed for strains in lanes 3, 6, 8 and 12, showing identical patterns consisting of fragments with molecular sizes corresponding to 1-20, 0-82, 0-75, 0-71, 0-35, 0-25 and 0-21 kbp. Strains in lanes 3 and 12 were isolates from sporadic cases in Iran, and those in lanes 6 and 8 were an isolate from France and strain ATCC 25763\textsuperscript{T}, respectively. Lanes 7 and 13 were isolates of sporadic cases in Iran and represented the third pattern with six fragments of molecular sizes of 1-20, 1-10, 0-48, 0-39, 0-33 and 0-25 kbp. The \textit{C. sporogenes} strains studied here were identified by the Rapid ID 32 A test strip (bioMérieux) and also tested negative for ABEFG neurotoxins by PCR. The results of \textit{HhaI} digestion of the rRNA genes of one representative of \textit{C. sporogenes} is shown in lane 5 with eight fragments with molecular sizes of 1-2, 1-1, 0-95, 0-81, 0-74, 0-43, 0-24 and 0-14 kbp. Some of the fragments of
C. sporogenes were, however, comparable to six C. botulinum strains A. All C. sporogenes isolates showed a similar restriction pattern.

**DISCUSSION**

It has been indicated that the strains in each group of C. botulinum are so closely related (Popoff, 1995) that no phenotypic distinction, including carbohydrate fermentation, between the toxigenic and non-toxigenic Clostridium strains can be achieved. For example, we have observed that the Rapid ID 32 A test cannot distinguish C. sporogenes from C. botulinum strains, in accordance with the report by Lindöstöm et al. (1999). Furthermore, the similarity between C. sporogenes and group I C. botulinum total DNA sequence and 16S rRNA gene is up to 90 and 95.4%, respectively (Popoff, 1995).

The problem of obtaining proper DNA for genotyping of C. botulinum has been indicated by several investigators and seems to be due to the production of extracellular DNases by bacteria and eventual DNA degradation during the isolation (Hielm et al., 1998). Therefore, this has greatly impaired the use of molecular biology typing methods and, furthermore, has made techniques such as PFGE typing more difficult (Hielm et al., 1998). In order to overcome this problem, we have utilized a rapid, ready-to-use DNA isolation kit, which gave reproducible results for typing of C. botulinum toxinotype A in ARDRA. However, it should be noted that the DNA prepared by this protocol may not be suitable for other genotyping techniques, as we could not obtain a proper ribotyping pattern using DNA prepared by the InstaGene purification matrix.

PCR-based DNA fingerprinting of micro-organisms has been described extensively using a wide variety of techniques and primer designs. Hyytiä et al. (1999a) evaluated RAPD analysis and repetitive element sequence-based PCR with respect to their applicability in characterizing C. botulinum group I and II strains. The authors were able to distinguish only group I strains at the serotype level only, suggesting that the genome of C. botulinum may not harbour repetitive sequences in large numbers or that group I of C. botulinum has a limited genetic diversity.

ARDRA has the advantage of speed of performance and ease of interpretation and was able to distinguish between C. botulinum of group I and C. sporogenes strains. The results indicated that, of the 10 restriction enzymes used, HhaI gave the most discriminatory restriction patterns for C. botulinum toxinotype A. ARDRA cluster analysis of the isolates confirmed the genetic diversity in C. botulinum toxinotype A strains, contrary to a report by Hyytiä et al. (1999a), who did not observe genetic diversity within group I C. botulinum using other techniques such as RAPD typing.

The 12 C. botulinum toxinotype A strains characterized in the present study were from an outbreak and sporadic cases in Iran, from a patient with botulism in France and an ATCC type strain. The two Iranian strains, the French strain and the ATCC type strain formed a group together, indicating close relatedness in these isolates from fairly diverse and different origins. The French isolate was collected about 20 years ago, whereas the Iranian strains were recent isolates. Overall, the results presented here suggest that, using ARDRA, correct genotyping of C. botulinum toxinotype A is possible. Furthermore, the genetic diversity observed among toxinotype A isolates indicates the value of this PCR-based technique as a rapid and reproducible tool that can be applied in the investigation of botulism. ARDRA of the 16S–23S rRNA genes has proved to be a rapid and technically simple method for recognizing strains belonging to C. botulinum toxinotype A.

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