AN ATYPICAL CLOSTRIDIUM STRAIN RELATED TO THE CLOSTRIDIUM BOTULINUM GROUP III ISOLATED FROM A HUMAN BLOOD CULTURE

Philippe BOUVET¹*, Raymond RUIMY², Christiane BOUCHIER³, Nathalie FAUCHER⁴, Christelle MAZUET¹, Michel R. POPOFF¹

¹ Institut Pasteur, Bactéries anaérobies et Toxines, Paris, France
² Centre Hospitalier Universitaire, Hôpital de l'Archet 2, Nice, France
³ Institut Pasteur, Plate-forme Génomique, Paris, France
⁴ Groupe Hospitalier Bichat Claude Bernard, Service de Gériatrie, Paris, France

Running Header: Clostridium botulinum, blood culture

Key Words: Clostridium botulinum, Clostridium novyi, Clostridium haemolyticum, blood culture, sepsis, multilocus sequence analysis.

* Corresponding author. Mailing address: Institut Pasteur, Unité Bactéries anaérobies et Toxines, 25-28 rue du Docteur Roux, F-75724 Paris cedex 15, France. Tel: +33 (0)1 40 61 35 09; Fax: +33 (0)1 40 61 31 23; E-mail: philippe.bouvet@pasteur.fr.
ABSTRACT

A non-toxigenic strain isolated from a fatal human case, was identified as a Clostridium from the C. botulinum group III based on the phenotypic characters and 16S rDNA sequence, and related to the mosaic C. botulinum D/C according to a multilocus sequence analysis of 5 housekeeping genes.

CASE REPORT

A 83-year-old woman was hospitalized in a hospital geriatric ward in Paris for a cardiac decompensation. She had a mental disorder and a transient motor weakness the day planned for her relapse, and she received a benzodiazepine treatment. The same day evening, she fell off her bed and had a fracture of the neck of the femur. Two days later, she developed sepsis (fever, hypotension 11/5, 33,000 leucocytes/mm$^3$, C reactive protein 30 mg/L) and received an antibiotic treatment (cefotaxime 2 g/d, metronidazole 0.5 g/8h iv). No characteristic symptom of flaccid paralysis was evidenced. Surgery was delayed and death occurred the following day. A blood culture performed during the septic phase yielded an anaerobic bacterium called strain AIP981.10.

Bacteria were grown in Trypticase yeast extract glucose haemin (TGYH) broth in an anaerobic atmosphere at 37°C. Phenotypic identification was performed with reference methods (1), and metabolic end products (volatile and non-volatile fatty acids) were assayed by quantitative gas chromatography as described previously (2). Toxicity was tested using mouse bioassay (3) and cytotoxicity on Vero cells was performed as previously described (4). 16S rDNA gene sequence was determined as described previously (5) and was compared to all eubacterial 16S rRNA gene sequences available in the GenBank database by using the multisequence Advanced BLAST comparison software from the National Center for Biotechnology Information (6).
Multilocus sequence analysis (MLST) was based on five housekeeping genes (CTPs, glpK, rpoB, gyrA, and dnaK) (Supplemental Table S1). Genome sequences of *C. botulinum* D strain 1873 (NZ_ACSJ01000007) were used as templates for sequence alignment of the clostridial genes, which have been analyzed, and primers were designed on the conserved sequences with Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

*C. botulinum* type A ATCC3502 and *C. perfringens* strain 13 were used as outgroups in gene analysis (Supplemental Table S2). In addition, botulinolysin, *C. botulinum* C2 toxin, *Clostridium novyi* hemolysin (termed novyiilysin) genes as well as flagellin genes from *C. novyi* A, B, *C. haemolyticum*, and *C. chauvoei* according to (7) were also investigated (Supplemental Table S1). Gene fragments were PCR amplified and sequenced. Sequence alignments and phylogenetic analysis were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software (version 5) (http://www.megasoftware.net) (8). The phylogenetic inference was based on the neighbor-joining distance method (9). Gene trees were constructed with the neighbor-joining method, using the Kimura two-parameter model (10) and bootstrapping algorithms contained in MEGA software (11).

Strain AIP981.10 was a strictly anaerobic, Gram-positive, spore-forming bacillus producing lipase and protease, but not lecithinase. Gas was produced. Catalase, urease, indole from tryptophan, reduction of nitrates and nitrites were found negative. Haemolysis on sheep blood agar was observed. A commercial gallery (rapid ID 32A, BioMérieux, Marcy l’Etoile, France) was inoculated and gave the resulting code 4006400000 that does not correspond to a known species. However, these results indicated that AIP981.10 might belong to the *C. botulinum/C. novyi/C. sporogenes* group of bacteria. Major volatile and non-volatile fatty acids were: propionic (35.7 mM), lactic (12.9 mM) and butyric (6.0mM) acids with little amounts of 2-hydroxy-butyric and 2-hydroxy-valeric acids. Production of propionic acid as major metabolism end products is characteristic of *C. botulinum* group III including *C.
botulinum C and D as well as related species such as C. novyi and C. haemolyticum, (1, 12)

Thus, AIP981.10 was tentatively assigned to a C. botulinum from group III or a related species.

Strain AIP981.10 was not toxic as monitored by injection of 1 ml of culture supernatant intraperitoneally into mice, and there was no cytotoxicity on Vero cells.

Botulinum neurotoxin (BoNT) A to G genes as well as flagellin genes of C. novyi A, B, C. haemolyticum, and C. chauvoei were not PCR detected. Among the toxin genes tested, AIP981.10 gave a PCR amplification only with botulinolysin primers (Table 1). Botulinolysin and novyilysin produced by C. botulinum and Clostridium novyi, respectively, are related hemolysins from the cholesterol-dependent cytolysin family, which also encompasses Clostridium tetani tetanolysin (13). Botulinolysin primers (Supplemental Table S1) yielded a PCR detection with all the C. botulinum, C. novyi and C. haemolyticum strains tested, whereas novyilysin primers were specific of C. novyi (Table 1) suggesting that AIP981.10 is more related to C. botulinum C, D, C. novyi B or C. haemolyticum than to C. novyi A. However, AIP981.10 did not contain C2 toxin genes. The 16S rDNA sequence from strain AIP981.10 (1,332 bp) clustered within Clostridium cluster I as defined by Collins et al. 1994 in the branch containing C. botulinum C, C. botulinum D, C. botulinum C/D and D/C mosaic, C. novyi and C. haemolyticum (Fig. 1) (7, 14, 15). Sequence from AIP981.10 was more related to those of C. botulinum C/D and C/D mosaic isolates (99.9% identity) compared to the other Clostridium sequences: C. botulinum C 468 (99.3%), C. botulinum D str. 1873 (99.6%), C. haemolyticum ATCC9650 (99.5%). C. novyi A (98.8) and C. botulinum C str. Eklund (98.1%).

Analysis of the five housekeeping genes and botulinolysin gene gave the same patterns for AIP981.10 as the 16s rDNA sequence phylogeny tree (Fig. 2). AIP981.10 is located on a distinct branch containing C. botulinum D/C mosaic strains between two closely related
branches one containing *C. botulinum* C/D strains and another one encompassing *C. novyi* B, *C. botulinum* C strain 468, *C. botulinum* D, and *C. haemolyticum*. Interestingly, the housekeeping gene sequences of *C. botulinum* C strain Eklund clustered within the branch containing *C. novyi* A, which is distantly related to that containing the *C. botulinum* C type strain 468. These results are in agreement with the phylogenomic analysis of Skarin et al. (16) which show that *C. botulinum* C strain Eklund is closely related to *C. novyi* A. The two *C. botulinum* C strains share 97.2% 16S rDNA sequence identity suggesting that strain Eklund might be assigned to a different species. Nucleotide differences in housekeeping gene and botulinolysin gene sequences between AIP981.10 and the *Clostridium* reference strains (Table 2) indicate that AIP981.10 is more related to the mosaic *C. botulinum* D/C strains than to the other *C. botulinum* and *C. novyi* strains.

In addition, whole genome sequencing of AIP981.10 has been performed with Illumina single-reads sequencing technology. Illumina library preparation and sequencing followed standard protocols developed by the supplier (TrueSeq DNA sample preparation). Briefly, genomic DNA was sheared by nebulization, and sheared fragments were end-repaired and phosphorylated. Blunt-end fragments were A-tailed, and sequencing adapters were ligated to the fragments. Fragments with an insert size of around 400 bp were gel-extracted and enriched with 10 cycles of PCR. Hybridization of the library to the flow cell and bridge amplification was performed to generate clusters, and single reads of 50 cycles were collected on a HiSeq 2000 (Illumina, San Diego, CA). After sequencing was complete, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline version 1.7. High-quality filtered reads were assembled using CLC Assembly Cell (CLC bio). 266 contigs ranging from 310 to 109,956 bp have been obtained. DNA sequences derived from standard PCR for 16s rDNA, botulinolysin genes and the five housekeeping genes were identical to those from the whole genome sequencing. Again, putative botulinum toxin gene
and C2 toxin genes have not been identified by sequence similarity searching. However, C3 exoenzyme and phospholipase C genes related to those found in \textit{C. botulinum} C and D (17, 18) have been evidenced in AIP981.10 (Table 1 and 2). An additional toxin gene, termed clostridiolysin S, has been recently characterized in \textit{C. botulinum}. Clostridiolysin S is related to Streptolysin S from \textit{Streptococcus} and is a post-translationally modified toxin resulting from a cluster of nine genes (\textit{closA, closB, closC, closD, closE, closF, closG, closH, closI}) (19). Among the \textit{C. botulinum}/\textit{C. sporogenes} whole genome sequences available, clostridiolysin S genes have been found only in \textit{C. botulinum} strains from group I and \textit{C. sporogenes}, whereas no related genes have been evidenced neither in \textit{C. botulinum} group III strains nor in AIP981.10.

Anaerobic bacteria are responsible for various types of infections including bacteremia. Blood stream infections with anaerobes (1 – 17\% of positive blood cultures) are mainly due to Gram-negative bacilli. \textit{Clostridium} account for 8 to 18\% (up to 46\% in some studies) of anaerobic bacteremia in adults [review in (20)]. The most frequently identified \textit{Clostridium} species are \textit{Clostridium perfringens} and \textit{Clostridium septicum} which are often associated with a dramatic outcome (20, 21). Other toxigenic Clostridia might also be involved such as \textit{Clostridium sordellii} (22), to a lower extent \textit{Clostridium difficile} (23), and one case of \textit{Clostridium tetani} (24). However, non-toxigenic Clostridia from the environment like \textit{Clostridium tertium}, \textit{Clostridium aldenense}, or \textit{Clostridium cadaveris}, are occasionally isolated in blood infections (25-27).

Here we report an atypical \textit{Clostridium} strain related to the \textit{Clostridium botulinum} group III, which has been identified in a blood culture. Phenotypic and genotypic analysis indicates that the strain AIP981.10 belongs to the physiological group III of \textit{C. botulinum} [reviewed in (28, 29)], which encompasses \textit{C. botulinum} type C, \textit{C. botulinum} type D, \textit{C. botulinum} mosaic C/D and D/C, and which is closely related to \textit{C. novyi} and \textit{C.
haemolyticum. Indeed, the chromosome of the group III C. botulinum isolates is highly conserved and is related to that of C. novyi (16). However, based on 16s rDNA sequence AIP981.10 is on a phylogenetic sub-branch distinct from those of C. botulinum and C. novyi strains, and multisequence analysis of housekeeping genes shows a close relatedness to C. botulinum D/C mosaic and to a lower extent to C. botulinum C/D mosaic. AIP981.10 which has been isolated from a blood culture of a fatal case was found to not produce lethal toxin. This strain contains botulinolysin, phospholipase C and C3 exoenzyme genes but not clostridiolysin S genes supporting that AIP981.10 is phylogenetically related to C. botulinum group III strains. In addition, the sequences of the botulinolysin and phospholipase C genes are highly related to those of C. botulinum C and D and less similar to those of C. novyi and C. haemolyticum (Table 2). BoNT genes type C and D as well as C. novyi alpha-toxin gene (tcnA) are localized on phage DNA which are not integrated into the chromosome. These phages can be easily lost upon subcultures and can be interchanged between C. botulinum C or D and C. novyi (30-34). Similarly, C2 toxin genes are localized on large plasmids in C. botulinum C and D which can be lost or acquired (35). It could be hypothesized that AIP981.10 has lost a phage harboring a bont gene during the isolation and subcultures of the strain, and is therefore no longer toxigenic. Indeed a C. botulinum type B-like nontoxigenic strain has been isolated from an infant botulism case (36). However, C. botulinum C or D have been involved only in a few cases of human botulism (37), and no characteristic symptoms of flaccid paralysis were observed in this patient. Alternatively, AIP981.10 could have lost either a phage harboring tcnA, a plasmid or another mobile genetic element carrying C2 toxin genes or other unknown toxin gene(s), since the group III C. botulinum strains possess various plasmids and mobile elements (35, 38). An environmental nontoxigenic Clostridium might also be the causative agent in compromised patients as already evidenced with other non-toxic Clostridium species (25-27).
176 16S rDNA, phospholipase C and C3 exoenzyme gene sequences from strain AIP981.10 were deposited in the GenBank database under the accession numbers KC589740, KF662728 and KF662729, respectively.

179 Acknowledgements

180 The authors thank S. Kozaki for kindly providing *C. botulinum* D/C isolates. This work was supported by Institut Pasteur funding.
REFERENCES


FIGURE LEGENDS

Figure 1. 16S rDNA analysis. Dendrogram was reconstructed from the nucleotide sequence of the gene by using the UPGMA method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates the genetic distance. The number shown next to each node indicates the percent bootstrap value of 1000 replicates evolutionary analyses were conducted in MEGA5 (8). Strain AIP981.10 is highlighted on the tree by using a black square ■.

Figure 2. Housekeeping gene and botulinolysin gene analysis. Dendrogram was reconstructed from the nucleotide sequence of the gene by using the neighbor-joining method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates the genetic distance. The number shown next to each node indicates the percent bootstrap value of 1000 replicates evolutionary analyses were conducted in MEGA5 (8). Strain AIP981.10 is highlighted on the trees by using a black square ■.
Figure 1
Table 1. PCR detection of novyilysin, botulinolysin, C2 toxin and clostridiolysin S genes in strain AIP981.10, *C. botulinum* C and D, *C. novyi* A and B, and *C. haemolyticum*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Novyilysin</th>
<th>Botulinolysin</th>
<th>Phospholipase C</th>
<th>C2 toxin component I</th>
<th>C2 toxin component II</th>
<th>C3 exoenzyme</th>
<th>Clostridiolysin S</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIP981.10</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>C. botulinum</em> C (str. 468)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>C. botulinum</em> D (str. 1873)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>C. botulinum</em> C/D mosaic</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>C. botulinum</em> D/C mosaic</td>
<td>-</td>
<td>+</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>C. haemolyticum</em> (AIP10052²)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>C. novyi</em> A (AIP10062¹)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. novyi</em> B (AIP212.86)</td>
<td>-</td>
<td>+</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A: not available
**Table 2.** Nucleotide differences between AIP981.10 gene sequences and corresponding sequences from *C. botulinum* C, D and C/D, *C. haemolyticum* and *C. novyi* NT.

<table>
<thead>
<tr>
<th>AIP981.10 genes (source)</th>
<th>Size (bp)</th>
<th>Number of nucleotide differences between AIP981.10 and corresponding <em>Clostridium</em> sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT35 (PCR amplicon)</td>
<td>466</td>
<td>0 10 19 49 50</td>
</tr>
<tr>
<td>glpK (PCR amplicon)</td>
<td>579</td>
<td>0 9/121 23 35/121 56</td>
</tr>
<tr>
<td>rpoB (PCR amplicon)</td>
<td>627</td>
<td>0 6 23 49 56</td>
</tr>
<tr>
<td>gyrD (PCR amplicon)</td>
<td>558</td>
<td>0 26 29 72 82</td>
</tr>
<tr>
<td>absA (PCR amplicon)</td>
<td>525</td>
<td>0 11/217 15 33 24/213 56</td>
</tr>
<tr>
<td>Botulinolysin (PCR amplicon)</td>
<td>902</td>
<td>2 26/154 176 168/195 210</td>
</tr>
<tr>
<td>Phospholipase C (gene sequence from WGS)</td>
<td>1200</td>
<td>N/A 55/234 74 / 77 / N/A / 105 388 394</td>
</tr>
<tr>
<td>C3 exoenzyme (gene)</td>
<td>735</td>
<td>N/A 16 16 / 1 / N/A / N/A 11 N/A</td>
</tr>
</tbody>
</table>
Some genomes show two gene copies of *dnaK*, *glpK*, botulinolysin and phospholipase C, that are distantly related (differences in both gene copies separated by a slash). The corresponding GenBank accession numbers of these genomes are listed in supplemental Table 2.

³ Data obtained from the whole genome sequencing (WGS) of strain AIP981.10.

³ Nucleotide difference between phospholipase C from AIP981.10 versus those from *C. botulinum* C (AB699605), *C. botulinum* D (AB699604), *C. novyi* B (N/A) and *C. haemolyticum* (AF525415), respectively.

¹ Nucleotide difference between C3 exoenzyme from AIP981.10 versus those from *C. botulinum* C (X59039), *C. botulinum* D (D17555), *C. novyi* B (N/A) and *C. haemolyticum* (N/A), respectively.

N/A: not available