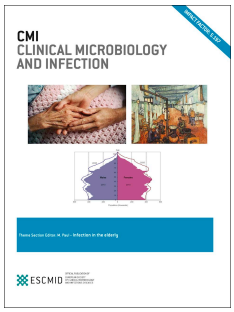
A penicillin and metronidazole resistant Clostridium botulinum strain responsible for an infant botulism case

Christelle Mazuet, Eun-Jeong Yoon, Sophie Boyer, Stéphanie Pignier, Thierry Blanc, Isabelle Doehring, Djalal Meziane-Cherif, Clémentine Dumant-Forest, Jean Sautereau, Christine Legeay, et al.

Accepted Manuscript



A penicillin and metronidazole resistant *Clostridium botulinum* strain responsible for an infant botulism case

Christelle Mazuet, Eun-Jeong Yoon, Sophie Boyer, Stéphanie Pignier, Thierry Blanc, Isabelle Doehring, Djalal Meziane-Cherif, Clémentine Dumant-Forest, Jean Sautereau, Christine Legeay, Philippe Bouvet, Christiane Bouchier, Suzana Quijano-Roy, Martine Pestel-Caron, Patrice Courvalin, Michel R. Popoff

PII: S1198-743X(16)30068-4

DOI: 10.1016/j.cmi.2016.04.011

Reference: CMI 577

To appear in: *Clinical Microbiology and Infection*

Received Date: 18 February 2016

Revised Date: 29 March 2016

Accepted Date: 8 April 2016

Please cite this article as: Mazuet C, Yoon E-J, Boyer S, Pignier S, Blanc T, Doehring I, Meziane-Cherif D, Dumant-Forest C, Sautereau J, Legeay C, Bouvet P, Bouchier C, Quijano-Roy S, Pestel-Caron M, Courvalin P, Popoff MR, A penicillin and metronidazole resistant *Clostridium botulinum* strain responsible for an infant botulism case, *Clinical Microbiology and Infection* (2016), doi: 10.1016/j.cmi.2016.04.011.

REVISED

A penicillin and metronidazole resistant *Clostridium botulinum* strain responsible for an infant botulism case

Christelle Mazuet,[1] Eun-Jeong Yoon,[2] Sophie Boyer,[3] Stéphanie Pignier,[4] Thierry Blanc,[5] Isabelle Doehring,[6] Djalal Meziane-Cherif,[2] Clémentine Dumant-Forest,[4] Jean Sautereau,[1] Christine Legeay,[1] Philippe Bouvet,[1] Christiane Bouchier,[7] Suzana Quijano-Roy,[6,8] Martine Pestel-Caron,[3] Patrice Courvalin,[2] and Michel R. Popoff[1]

[1]Institut Pasteur, Unité des Bactéries anaérobies et Toxines, Paris, France

[2]Institut Pasteur, Unité des Agents Antibactériens, Paris, France

[3]Hôpital Charles Nicolle, Département de Microbiologie, GRAM (EA2656), Rouen, France

[4]Hôpital Charles Nicolle, Pédiatrie médicale, Rouen, France

[5]Hôpital Charles Nicolle, Pédiatrie néonatale et réanimation, Rouen, France

[6]AP-HP, Service de Pédiatrie-Réanimation, Pôle Pédiatrique, Hôpital R. Poincaré, Garches. Hôpitaux Universitaires Paris-Ile-de-France Ouest, UVSQ, France.

[7]Institut Pasteur, Plateforme Genomique-Pôle Biomics, Paris, France

[8]Centre de Référence des Maladies Neuromusculaires GNMH (FILNEMUS), France

CM[1] and EJY[2] contributed equally to this work

Correspondence to:

Michel R. Popoff

Institut Pasteur, Unité des Bactéries anaérobies et Toxines,

25-28, rue du Docteur Roux 75724 Paris Cedex 15, France

Tel : +33 (0)1 45 68 83 07

Fax : +33 (0)1 40 61 31 23

Email : michel-robert.popoff@pasteur.fr

Key Words: Clostridium botulinum, botulism, infant botulism, antibiotic-resistance, penicillin, metronidazole

ABSTRACT

An infant botulism clinical course was characterized by several relapses despite therapy with amoxicillin and metronidazole. Botulism was confirmed by identification of botulinum toxin and *C. botulinum* in stools. A *C. botulinum* A2 strain resistant to penicillins and with heterogeneous resistance to metronidazole was isolated from stool samples up to 110 days after onset. Antibiotic susceptibility was tested by disk agar diffusion and minimum inhibitory concentrations were determined by Etest. Whole genome sequencing allowed detection of a gene cluster composed of bla_{CBP} for a novel penicillinase, *blaI* for a regulator, and *blaR1* for a membrane bound penicillin receptor in the chromosome of the *C. botulinum* isolate. The purified recombinant penicillinase was assayed. Resistance to β-lactams was in agreement with the kinetic parameters of the enzyme. In addition, the β-lactamase gene cluster was found in three *C. botulinum* genomes in databanks and in two out of 62 genomes of our collection, all the strains belonging to group I *C. botulinum*. This is the first report of a *C. botulinum* isolate resistant to penicillins. This stresses the importance of antibiotic susceptibility testing for adequate therapy of botulism.

INTRODUCTION

Botulinum neurotoxins (BoNTs) are the most potent toxins known and are responsible for severe neurological disorder in man and animals. Botulism is acquired by ingestion of preformed BoNT in food (foodborne botulism), or after intestinal (infant botulism, adult intestinal toxemia botulism) or wound (wound botulism) colonization and in situ BoNT production [1]. Infant botulism occurs between two weeks and one year of age and results from ingestion of *Clostridium botulinum* spores or bacteria, subsequent clostridial growth and toxin production in the intestine, and finally passage of BoNT through the intestinal mucosa to motornerve endings. Infant botulism is common in some countries and certain states of the USA [1-3] but is rarely reported in Europe [4]. In France, foodborne botulism is the main form of the disease whereas only a few cases of infant botulism have been identified [5].

BoNTs are divided into seven toxinotypes (A to G) according to their immunological properties and in numerous subtypes based on amino acid sequence variations [6]. A new BoNT type called H has been reported but was characterized as an A/F hybrid [7, 8]. BoNTs are produced by heterogeneous groups of *Clostridium* including *C. botulinum* and atypical strains of other species such as *Clostridium baratii* and *Clostridium butyricum* [6]. Like other *Clostridium* species and anaerobes, *C. botulinum* is intrinsically resistant to aminoglycosides, to sulfamethoxazole and trimethoprim but remains susceptible to other drug classes [9, 10]. However, only a small number of strains have been tested for susceptibility to antibiotics [9, 10]. Antibiotics are frequently used for presumed sepsis [11, 12]. but might exacerbate botulinum symptoms [4, 11]. When indicated, β-lactams are the antibiotics of choice for clostridial infections [12]. We report the characterization, to the best of our knowledge, of the first *C. botulinum* strain resistant to β-lactams and responsible for an infant botulism case,

albeit other *C. botulinum* strains isolated before contained uncharacterized β-lactamase gene (see below). The isolate also displayed diminished susceptibility to metronidazole.

MATERIALS AND METHODS

Ethics Statement

All experiments were performed in accordance with the French and European Community guidelines for laboratory animal handling (agreement of laboratory animal use n° 2013-0116).

DNA Preparation, Recombinant DNA Techniques, Protein Preparation

Total DNA was isolated from *C. botulinum* as described [13]. DNA extraction from stool samples was performed with DNA stool kit (Qiagen) according to the manufacturer's recommendations. Detection of *C. botulinum* in biological samples was performed by SYBR-green real-time PCR with specific primers as previously described [13]. The bla_{CBP} gene was amplified with primers P2251 (GGATCCATGAAAAAAATAGTAAACTC) and P2252 (GTCGACTATTTCCTGGTGTTAATAAA) adding *BamH*I and *Sal*I sites (underlined), and cloned into pET28a. The resulting plasmid introduced into *E. coli* BL21(DE3) was verified by DNA sequencing. The *C. botulinum* penicillinase (CBP) with a N-terminal 6-His tag was produced and purified as described [14].

Toxin Detection

Toxin detection and titration in biological samples or in culture supernatants were performed by the mouse bioassay with specific neutralizing antibodies [15]. Half ml of ten-fold serial dilutions of samples in 50 mM phosphate buffer (pH 6.5) containing 1% gelatin were injected intraperitoneally into Swiss mice weighing 20-22 g (Charles River).

Whole Genome Sequencing (WGS)

The WGS libraries performed using the NEBNext Ultra DNA Library Prep kit for Illumina (New England Biolabs) were sequenced on MiSeq or HiSeq2000 machines (Illumina). Sequence files were generated using Illumina Analysis Pipeline version 1.8 (CASAVA). After quality filtering, reads were assembled using CLC software version 4 (CLC Bio).

RESULTS

Case Report

On February 21st 2013, a 2-month-old girl was hospitalized after 24 h of progressive floppiness and feeding difficulties. She rapidly developed a profound hypotonia with absent suckling reflex, lethargy, and required mechanical ventilation. Ionogram, cerebrospinal fluid, electroencephalography, and encephalic magnetic resonance imaging were normal. Three Hz repetitive stimulation did not show decremental muscle response and there was no clinical improvement with anticholinesterasics, ruling out a post-synaptic myasthenic disorder. Myopathy was suggested by initial electromyogram (EMG) findings but a muscle biopsy was normal. The symptoms persisted and a second EMG 17 days later revealed spontaneous activity and early nerve regeneration potentials suggesting a disorder associated with acute nerve denervation and regeneration, but intravenous immunoglobulins had no obvious effect. Although no facilitation was observed on high frequency repetitive stimulation under sedation (20 and 50 Hz), botulism was investigated due to the descendent progressive tetraplegia with predominance of facial, ocular and bulbar paralysis, mydriasis and persistent constipation. The first stool and serum samples were taken 25 and 28 days after the onset of clinical signs (Figure and Table S1 in the Supplementary Appendix). The baby received amoxicillin (50 mg/Kg, 3 times per day intravenously) and metronidazole (40 mg/Kg in 3 intravenous administrations per day) during 10 days. After 34 days of

hospitalization she improved and was discharged. Eleven days later she was re-hospitalized for hypotonia, absence of suckling, respiratory distress, closed eyes and was treated with 10 mL of trivalent (anti ABE) equine antitoxin (Behring) associated with amoxicillin and metronidazole at the same posology for eight days by intravenous route and two additional days by oral route. A rapid improvement was observed two days after the anti-toxin and antibiotic administration. The baby was fed with women milk and received probiotics (Biogaïa® 5 drops a day, Lactéol® 2 bulbs a day) during 15 days. Constipation and suckling difficulties persisted 74 days after the onset but her global clinical status clearly improved. Nevertheless, she was again hospitalized due to the persistence of BoNT and *C. botulinum* in stool samples (see below). The baby received vancomycin (15 mg/Kg in 3 oral administrations per day) during 10 days, and recovered gradually of her generalized weakness. The main steps of the clinical course, chronology of the biological samples and BoNT/A titration and PCR detection of *C. botulinum* A in stool samples are summarized in Figure and Table S1 in the Supplementary Appendix. BoNT/A was detected in stools at variable concentrations according to the clinical phases with high levels during the two relapses but not in serum. *C. botulinum* A was found in stools up to 114 days after the onset of the symptoms (Figure and Table S1 in the Supplementary Appendix)

Antibiotic Susceptibility of *C. botulinum* Strains

The six *C. botulinum* strains isolated from stool samples (Figure and Table S1 in the Supplementary Appendix) produced BoNT/A. Whole genome sequencing of these strains indicated that they were identical. The strains were assigned to subtype A2 based on the deduced amino acid sequences of *bont/A* genes and to the multi locus sequence typing (MLST) profile 22 [16]. Strain 224-13 was selected for further studies.

In addition to *C. botulinum* intrinsic resistance to trimethoprim/sulfamethoxazole and aminoglycosides [17] the strain 224-13 was resistant to penicillin G, amoxicillin, ticarcillin, mezlocillin, and cephalothin but remained susceptible to other antibiotics including vancomycin (Table 1). The MICs against strain 224-13 confirmed high-level resistance (MIC >256 µg/mL) to penicillins and to cephalothin. Moreover, inducible, heterogeneous, and reversible resistance to metronidazole was observed in strain 224-13 as already described for *C. difficile* [18]. Colonies grew inside the inhibition zone after 48 h of incubation with MICs ranging from 1 to >256 µg/mL. It is noteworthy that albeit the agar disk diffusion method is not recommended by the CLSI (Clinical & Laboratory Standards Institute), the diffusion method with metronidazole disk (5 µg disk) allowed the detection of metronidazole heterogeneous resistance in *C. difficile* [18] as well as in *C. botulinum* (this study). A *nim* nitroimidazole reductase gene was found in the genome of 224-13 as in most *C. botulinum* group I strains which remain susceptible to the drug. However, this gene, when expressed, is responsible for high-level resistance to metronidazole and no typical promoter was found upstream indicating that it was not involved in heterogeneous resistance. Indeed, *nim*-independent metronidazole resistance has already been observed in other bacteria such as *Bacteroides* and *C. difficile* [18, 19]. Heterogeneous resistance to metronidazole might lead to recurrence or clinical failure as observed in *C. difficile* infections [20].

C. botulinum 224-13 Contains a Functional β-lactamase Gene Cluster

Blast analysis of strain 224-13 genome revealed the presence of a gene for a putative β-lactamase related to that of *Bacillus cereus* (54% identity), *Bacillus licheniformis* (48%), and *Staphylococcus aureus* (40%) which was assigned to a novel family of class A enzymes designated CBP for *C. botulinum* penicillinase. Two genes related to the regulatory genes *blaI* and *blaR1* in *B. cereus* and *S. aureus* [21] were found downstream

from *blaP*. In contrast to the cluster organization in these two species, in which the β-lactamase gene is transcribed in opposite direction to that of *blaI* and *blaR1,* the genes from strain 224-13 were in the same orientation. The base composition of the gene cluster was 27.5% similar to those of the chromosome of *C. botulinum* (mol% G+C 28%) and *C. butyricum* (29%).

The bla_{CBP} gene cloned into *E. coli* conferred resistance to penicillins and susceptibility was restored by addition of β-lactamase inhibitors clavulanic acid and sulbactam (Table 2). Benzylpenicillin was the best substrate for the enzyme with a high catalytic efficiency (k_{cat}/K_m, 2907 mM^{-1} s^{-1}). CBP also hydrolyzed cephalothin with a catalytic efficiency 2.4-fold lower than that of benzylpenicillin, but was not active on ceftazidime and cefoxitin even at a high enzyme concentration (1 μM). Aztreonam was very weakly hydrolyzed and no activity was observed against imipenem (Table 3). These data confirmed that bla_{CBP} was responsible for β-lactam resistance in *C. botulinum* and were in agreement with the resistance phenotype.

The β-lactamase Gene Cluster is Present in Various *C. botulinum* Strains

In strain 224-13, the β-lactamase gene cluster was located in a 433 kb contig that also carried genes for ribosomal protein S5, and for RNA polymerase sigma factor indicating a chromosomal location. We did not find any known insertion sequence nor any prophage in the 200 kb vicinity of the cluster. Blast analysis of twenty complete genomes of *C. botulinum* in GenBank (last access, September 2015) with the bla_{CBP}, *blaI*, and *blaR1* sequences from 224-13 showed that *C. botulinum* Kyoto F [22] and H04402 065 [23] contained identical or closely related clusters (Table 4 and Figure S1 in the Supplementary Appendix). The cluster was also found in one additional strain with partially sequenced genome, *C. botulinum* F CDC54085. In addition, screening of a total of 62 genomes in our strain collection revealed that *C. botulinum* 301-13 responsible for

foodborne botulism and 126-07 isolated from wound botulism also harbored the chromosomal cluster, and both isolates were resistant to penicillins (Table S2 in the Supplementary Appendix).

The genomic environment of the cluster was studied by comparing the flanking regions from *greA* to *nifE* of *C. botulinum* 224-13 with those of strains of various toxin types (Figure S2 in the Supplementary Appendix). The *ca.* 20 kb region was identical in 224-13 and Kyoto F, and had 99.8% identity between 125-07 and H004402 065. All the strains had two conserved regions (CR): CR left (CRL) from *greA* to ORF1, and CR right (CRR) between *araC* and *nifE*. While Loch Maree only possessed these two regions, other strains had insertion of a Type I DNA restriction modification system composed of *hsdM*, *S*, and *R* genes between the two CRs. Subsequent insertions likely occurred in two loci: i) of various ORFs of unknown function between *hsdS* and *R* and ii) between restriction modification system and the CRR, the β-lactamase cluster and two upstream genes *tfoX* for the C-terminal portion of competence protein TfoX and *gyrI* for a DNA gyrase inhibitor, and downstream a gene for part of a transposase. In strain Hall the gene cluster was replaced by *pcrA* for a putative ATP-dependent DNA helicase, and the same sequence was present in *C. botulinum* ATCC 19397 and ATCC 3502, also of A1 subtype.

DISCUSSION

Infant botulism is characterized by intestinal colonization of infants less than 12 months of age by *C. botulinum* and in situ toxin production. Excretion of BoNT and *C. botulinum* in the feces and, more rarely, the presence of BoNT in the serum are common features of the disease. The persistence of *C. botulinum* in stool samples usually varies from 2 to 4 months [24, 25]. Prolonged fecal excretion of *C. botulinum* type A up to 5 months after the onset of clinical symptoms has been reported [26]. In the present case, *C. botulinum*

was detected in the stools up to 110 days (Figure and Table S1 in the Supplementary Appendix). BoNT/A was present in the stool samples concomitantly with *C. botulinum* but at varying levels which might reflect phases of clostridial growth and toxin production. The BoNT levels in stools could be related to the various clinical phases accompanied by constipation.

The origin of this infant botulism case has not been identified. The baby did not receive any food known to be at risk for botulism, such as honey [25], but lived close (about 200 m) to a thermal power station that intermittently releases sprays of vapour and smoke/dust. The environment seems to be the likely source of contamination by dispersion of *C. botulinum* spores in the environment of the baby, as previously reported in Finland where *C. botulinum* was recovered in household dust [26] .

The most striking feature was resistance of the *C. botulinum* A2 isolates to penicillins and metronidazole which accounts for the failure of intestinal elimination of *C. botulinum* by the first two treatments with a combination of both drugs. After vancomycin treatment from day 76 to 84, *C. botulinum* and BoNT/A were still detected up to day 114 in the stool samples (Figure and Table S1 in the Supplementary Appendix). To the best of our knowledge, this is the first report of β-lactam resistance in *C. botulinum*. A previous study showed that, like all other *Clostridium* species, *C. botulinum* strains from infant botulism or wound botulism are as susceptible as environmental strains to all the antibiotics tested except sulfamethoxazole/trimethoprim and aminoglycosides [10]. In contrast, penicillin resistance has been reported in *C. butyricum* from neonatal necrotizing enterocolitis [27, 28] and up to 15% of the strains are resistant [29]. The MICs of penicillin G and piperacillin against *C. butyricum* have been reported to raise up to 64 µg/mL [29], whereas a much higher level of resistance (>256 µg/mL) was observed for the *C. botulinum* strains in this study. Other clostridia may

produce ß-lactamase including *Clostridium clostridioforme*, *Clostridium ramosum*, and *Clostridium innocuum* but the action of ß-lactamase inhibitor and substrate profile of hydrolysis were different [30, 31].

Whole genome sequencing of the *C. botulinum* isolates from the infant botulism case indicated the persistence of a single isolate and showed the presence of novel β-lactamase gene *bla*$_{CBP}$ and of downstream genes *blaI*, and *blaR1* homologous to those of *Bacillus* spp. and *S. aureus* [21, 29]. Cloning of *bla*$_{CBP}$ from strain 224-13 into *E. coli* and kinetic of CBP (Table 3) confirmed that the gene is responsible for resistance to penicillins and that CBP belongs to a novel class A β-lactamase. Overall, kinetic parameters were in agreement with the resistance phenotype of the hosts indicating that, as with class A enzymes, penicillin G was the best substrate. However, the penicillinase activity was approximately 30-fold lower than that of TEM-1 class A β-lactamase, but similar to that of *Streptomyces albus* G serine β-lactamase [32]

The deduced sequences of *bla*$_{CBP}$, *blaI,* and *blaR1* were more closely related to those of the orthologues in *C. butyricum* than to those in *B. cereus* and to a lower extent in *S. aureus* (Table 4). This suggests that the genes could derive from a common ancestor with a distinct evolution in *Clostridium* and in other Gram-positive bacteria. This is further supported by the base composition of the *C. botulinum bla* gene cluster which is similar to that of *Clostridium* genomes and distinct from those of *Bacillus* and *Staphylococcus.* In neurotoxigenic *C. butyricum*, the β-lactamase gene was found to be located on a linear megaplasmid (>610 kb) [33] a localization which might support its wide distribution in this species although transfer of the plasmid has not yet been documented. In contrast, the penicillinase gene cluster was located in the chromosome of strain 224-13 and of the other penicillin resistant *C. botulinum* A strains.

Interestingly, the β-lactamase cluster was found in *C. botulinum* strains of various BoNT type and subtypes that all belong to group I. The most likely evolution is acquisition by Loch Maree of the DNA restriction modification system leading to strains 429-13, 1430-11, and 1141-11 followed by acquisition of the penicillinase gene cluster by Kyoto F, 224-13, 301-13, H04402 065, and 126-07 while Hall acquired *pcrA*. Insertion of the cluster or of *pcrA* occurred upstream from *araC* which could constitute a hot spot for integration in *C. botulinum* group I strains which encompasses *C. botulinum* A and proteolytic *C. botulinum* B and F strains [6]. The cluster is flanked downstream by a truncated transposase gene and upstream by genes *tfoX* and *gyrI* which encode putative proteins involved in DNA transformation and rearrangement, respectively. It can thus be hypothesized that the gene cluster was part of a mobile DNA element. This evolution scheme is only speculative, more complex DNA rearrangements could have occure. For example, acquisition of the penicillinase gene cluster could be an older event and this cluster could have been replaced by *pcrA* such as in Hall strain.

In conclusion, we report an infant botulism case with prolonged excretion of a *C. botulinum* A2 strain resistant to penicillins and to low levels of metronidazole. Antibiotics could be useful to eradicate *C. botulinum* from the intestine but are considered to be responsible for exacerbation of the symptoms [4, 11]. This observation stresses that, when antibiotics are indicated, susceptibility of *C. botulinum* should be investigated for appropriate therapy of patients with botulism.

Acknowledgments

We thank M. Tichit for technical assistance. Sequencing was performed at the Genomics Platform, member of "France Génomique" consortium (ANR10-INBS-09-08). This publication used the *Clostridium botulinum* MLST website (http://pubmlst.org/cbotulinum) developed by K. Jolley, University of Oxford, developed and funded by the Wellcome Trust.

This work was supported by Institut Pasteur, Institut national de Veille Sanitaire, and by an unrestricted grant from Reckitt Benckiser.

REFERENCES

1 Sobel J. Botulism. *Clin Infect Dis*. 2005; **41**: 1167-1173.

2 Arnon SS. Infant botulism: Anticipating the second decade. *J Infect Dis*. 1986; **154**: 201-206.

3 Dabritz HA, Hill KK, Barash JR, et al. Molecular epidemiology of infant botulism in California and elsewhere, 1976-2010. *J Infect Dis*. 2014; **210**: 1711-1722.

4 Fenicia L, Anniballi F. Infant botulism. *Ann Ist Super Sanita*. 2009; **45**: 134-146.

5 King LA, Niskanen T, Junnikkala M, et al. Botulism and hot-smoked whitefish: A family cluster of type E botulism in france, september 2009. *Euro Surveill*. 2009; **14**.

6 Hill KK, Smith TJ. Genetic diversity within *Clostridium botulinum* serotypes, botulinum neurotoxin gene clusters and toxin subtypes. *Curr Top Microbiol Immunol*. 2013; **364**: 1-20.

7 Barash JR, Arnon SS. A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins. *J Infect Dis*. 2014; **209**: 183-191.

8 Maslanka SE, Luquez C, Dykes JK, et al. A novel botulinum neurotoxin, previously reported as serotype H, has a hybrid-like structure with regions of similarity to the structures of serotypes a and f and is neutralized with serotype A antitoxin. *J Infect Dis*. 2016; **213**: 379-385.

9 Dezfulian M, Dowell VR, Jr. Cultural and physiological characteristics and antimicrobial susceptibility of *Clostridium botulinum* isolates from foodborne and infant botulism cases. *J Clin Microbiol*. 1980; **11**: 604-609.

10 Swenson JM, Thornsberry C, McCroskey LM, Hatheway CL, Dowell VR, Jr. Susceptibility of *Clostridium botulinum* to thirteen antimicrobial agents. *Antimicrob Agents Chemother*. 1980; **18**: 13-19.

11 Fox CK, Keet CA, Strober JB. Recent advances in infant botulism. *Pediatr Neurol*. 2005; **32**: 149-154.

12 Nord CE, Hedberg M. Resistance to beta-lactam antibiotics in anaerobic bacteria. *Rev Infect Dis*. 1990; **12**: S231-234.

13 Vanhomwegen J, Berthet N, Mazuet C, et al. Application of high-density DNA resequencing microarray for detection and characterization of botulinum neurotoxin-producing clostridia. *PLoS One*. 2013; **8**: e67510.

14 Meziane-Cherif D, Lambert T, Dupechez M, Courvalin P, Galimand M. Genetic and biochemical characterization of oxa-63, a new class D beta-lactamase from *Brachyspira pilosicoli* bm4442. *Antimicrob Agents Chemother*. 2008; **52**: 1264-1268.

15 Mazuet C, Dano J, Popoff MR, Creminon C, Volland H. Characterization of botulinum neurotoxin type A neutralizing monoclonal antibodies and influence of their half-lives on therapeutic activity. *PLoS One*. 2010; **5**: e12416.

16 Jacobson MJ, Lin G, Raphael B, Andreadis J, Johnson EA. Analysis of neurotoxin cluster genes in *Clostridium botulinum* strains producing botulinum neurotoxin serotype A subtypes. *Appl Environ Microbiol*. 2008; **74**: 2778-2786.

17 Dezfulian M, McCroskey LM, Hatheway CL, Dowell VR, Jr. Selective medium for isolation of *Clostridium botulinum* from human feces. *J Clin Microbiol*. 1981; **13**: 526-531.

18 Pelaez T, Cercenado E, Alcala L, et al. Metronidazole resistance in *Clostridium difficile* is heterogeneous. *J Clin Microbiol*. 2008; **46**: 3028-3032.

19 Gal M, Brazier JS. Metronidazole resistance in *Bacteroides* spp. Carrying nim genes and the selection of slow-growing metronidazole-resistant mutants. *J Antimicrob Chemother*. 2004; **54**: 109-116.

20 Sanchez-Somolinos M, Alcala L, Pelaez T, et al. High levels of resistance to fluoroquinolones among *Clostridium difficile* isolates in a spanish hospital. *Clin Infect Dis*. 2008; **47**: 818-822.

21 Amoroso A, Boudet J, Berzigotti S, et al. A peptidoglycan fragment triggers beta-lactam resistance in *Bacillus licheniformis*. *PLoS Pathog*. 2012; **8**: e1002571.

22 Dineen SS, Bradshaw M, Karasek CE, Johnson EA. Nucleotide sequence and transcriptional analysis of the type A2 neurotoxin gene cluster in *Clostridium botulinum*. *FEMS Microbiol Lett*. 2004; **235**: 9-16.

23 Carter AT, Pearson BM, Crossman LC, et al. Complete genome sequence of the proteolytic *Clostridium botulinum* type A5 (B3') strain h04402 065. *J Bacteriol*. 2011; **193**: 2351-2352.

24 Fenicia L, Da Dalt L, Anniballi F, Franciosa G, Zanconato S, Aureli P. A case of infant botulism due to neurotoxigenic *Clostridium butyricum* type E associated with *Clostridium difficile* colitis. *Eur J Clin Microbiol Infect Dis*. 2002; **21**: 736-738.

25 Grant KA, McLauchlin J, Amar C. Infant botulism: Advice on avoiding feeding honey to babies and other possible risk factors. *Community Pract*. 2013; **86**: 44-46.

26 Derman Y, Korkeala H, Salo E, Lonnqvist T, Saxen H, Lindstrom M. Infant botulism with prolonged faecal excretion of botulinum neurotoxin and *Clostridium botulinum* for 7 months. *Epidemiol Infect*. 2014; **142**: 335-339.

27 Hart CA, Barr K, Makin T, Brown P, Cooke RW. Characteristics of a beta-lactamase produced by *Clostridium butyricum*. *J Antimicrob Chemother*. 1982; **10**: 31-35.

28 Magot M. Some properties of the *Clostridium butyricum* group beta-lactamase. *J Gen Microbiol*. 1981; **127**: 113-119.

29 Ferraris L, Butel MJ, Aires J. Antimicrobial susceptibility and resistance determinants of *Clostridium butyricum* isolates from preterm infants. *Int J Antimicrob Agents*. 2010; **36**: 420-423.

30 Alexander CJ, Citron DM, Brazier JS, Goldstein EJ. Identification and antimicrobial resistance patterns of clinical isolates of *Clostridium clostridioforme*, *Clostridium innocuum*, and *Clostridium ramosum* compared with those of clinical isolates of clostridium perfringens. *J Clin Microbiol*. 1995; **33**: 3209-3215.

31 Philippon A, Slama P, Deny P, Labia R. A structure-based classification of class A beta-lactamases, a broadly diverse family of enzymes. *Clin Microbiol Rev*. 2016; **29**: 29-57.

32 Matagne A, Lamotte-Brasseur J, Frere JM. Catalytic properties of class a beta-lactamases: Efficiency and diversity. *Biochem J*. 1998; **330 (Pt 2)**: 581-598.

33 Franciosa G, Scalfaro C, Di Bonito P, Vitale M, Aureli P. Identification of novel linear megaplasmids carrying a beta-lactamase gene in neurotoxigenic *Clostridium butyricum* type E strains. *PLoS One*. 2011; **6**: e21706.

34 Cinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. Wayne, PA 2015.

Table 1. Antibiotic resistance of *C. botulinum* strain 224-13.

Antibiotic	Clinical categorization by disk diffusion*	MIC (µg/mL) by Etest†
Penicillin G	R	>256
Amoxicillin	R	>256
Amoxicillin/clavulanic acid	S	1.5
Ticarcillin	R	ND
Mezlocillin	R	ND
Piperacillin	ND	>256
Piperacillin/Tazobactam	ND	3
Cephalothin	R	>256
Cefoxitin	S	ND
Cefotaxime	S	1.5
Imipenem	S	ND
Moxalactam	S	ND
Clindamycin	I	ND
Erythromycin	S	ND
Rifampicin	I	ND
Vancomycin	S	ND
Chloramphenicol	I	ND
Moxifloxacin	S	ND
Kanamycin	R	ND
Trimethoprim/sulfamethoxazole	R	ND
Metronidazole	HR	ND

HR, heterogeneous resistance; I, intermediate; ND, not determined; R, resistant; S, susceptible.

*Antibiotic susceptibility was tested by disk (Bio-Rad) diffusion on Wilkins-Chalgren agar according to the standards of the Comité de l'Antibiogramme de la Société Française de Microbiologie (http://www.sfm-microbiologie.org).

†The minimal inhibitory concentrations (MICs) of antibiotics were determined by Etest (bioMérieux) according to the manufacturer's recommendations.

Table 2. MICs of β-lactams against *E. coli.*

Antibiotic	**MIC (μg/mL)* against : *E. coli* BL21 (DE3) harboring**	
	pET28	**pET28Ω*bla*$_{CBP}$**
Amoxicillin	2	16
Amoxicillin/clavulanic acid	2	2
Ampicillin	0.5	32
Ampicillin/sulbactam	1	2
Piperacillin	1	8
Piperacillin/tazobactam	1	1
Ticarcillin	1	128
Ticarcillin/clavulanic acid	1	1
Cephalothin	1	2
Cefotaxime	0.015	0.015

*MICs were determined by microdilution according to the CLSI guidelines [34]

Table 3. Kinetic parameters of purified CBP β-lactamase for hydrolysis of β-lactams.*

Substrate	K_M (µM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1}) $\times 10^3$	Relative k_{cat}/K_M
Penicillin G	160 ± 50	465.2 ± 42.2	2.9 ± 1.2	100.0
Ticarcillin	140 ± 30	256.4 ± 34	1.8 ± 0.6	62.9
Ampicillin	530 ± 170	1 326.4 ± 317.3	2.5 ± 1.4	86.0
Amoxicillin	810 ± 150	215.8 ± 60.3	0.3 ± 0.1	9.2
Cephalothin	140 ± 20	169.7 ± 17.1	1.2 ± 0.3	41.6
Cefoxitin	NA	ND	NA	NA
Ceftazidime	NA	ND	NA	NA
Aztreonam†	NA	>18.2 ± 2.7	NA	NA
Imipenem	NA	ND	NA	NA

NA, not applicable; ND, not determined.

*Data are the means of three independent determinations. Relative values were calculated according to that obtained for benzylpenicillin which was set at 100.

†k_{cat} was estimated at high aztreonam concentration (1.4 mM) and did not enable to determine the K_M.

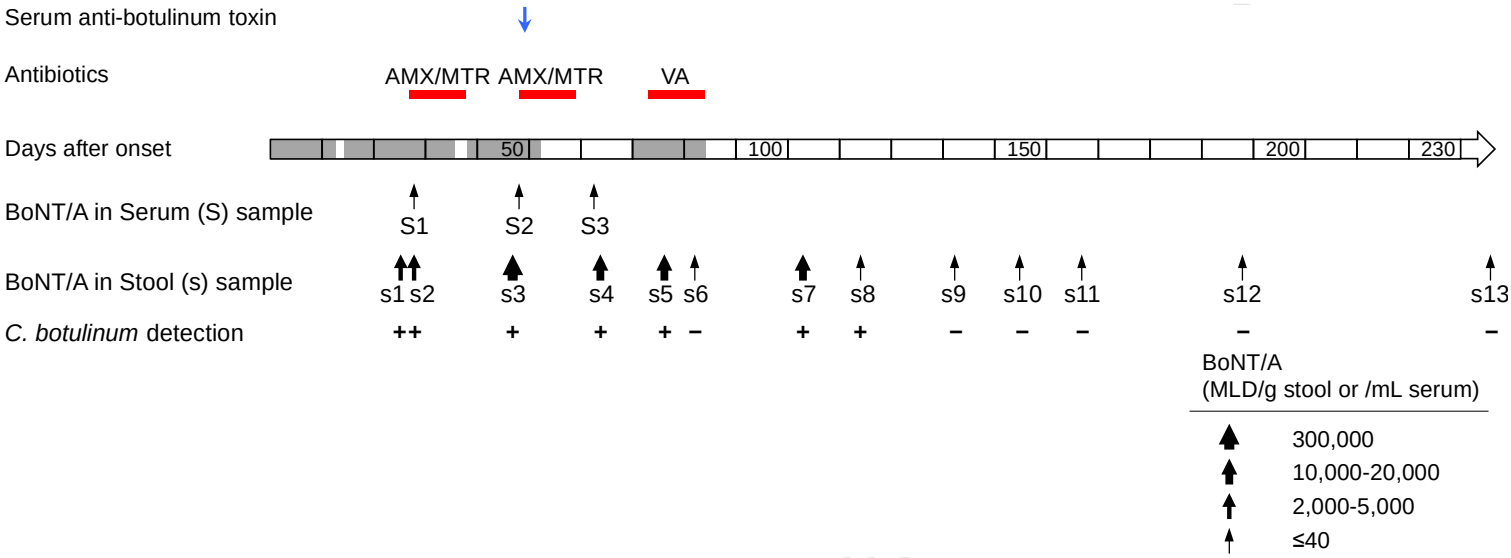
Table 4. Percent identity between Bla proteins versus those from strain 224-13.

Strain	CBP	BlaI	BlaR1
C. botulinum A2 Kyoto F	100	100	100
C. botulinum A2 301-13	98	98	96
C. botulinum A5 H04402 065	98	98	98[a]
C. botulinum A5 126-07	98	98	96
C. butyricum 60 E.3	71	67	67
B. cereus VD078/VDM022/CD160	54	48	29*
B. licheniformis ATCC 14580	48	43	33
S. aureus DAR3892	40	42	34

CBP, *Clostridium botulinum* penicillinase

The accession numbers of the sequences of strain 224-13 are KP718480 (bla_{CBP}), KP718481 (*blaR1*) and KP718482 (*blaI*). The CBP sequence has been submitted to http://www.ncbi.nlm.nih.gov/projects/pathogens/submit_beta_lactamase/.

*Identity of partial amino acid sequences.



LEGEND TO THE FIGURE

Figure. Patient's samples and therapy.

Grey box, hospitalization; red line, antibiotic treatment. Antibiotics: AMX, amoxicillin; MTR, metronidazole; VA, vancomycin. MLD, mouse lethal dose.