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Original article

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

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ABSTRACT

The clinical course of a case of infant botulism was characterized by several relapses despite therapy with amoxicillin and metronidazole. Botulism was confirmed by identification of botulinum toxin and *Clostridium 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 disc agar diffusion and MICs 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 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. **C. Mazuet, CMI 2016;22:644.e7–644.e12**

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 (food-borne botulism), or after intestinal (infant botulism, adult intestinal toxaemia botulism) or wound (wound botulism) colonization and *in situ* BoNT production [1]. Infant botulism occurs between 2 weeks and 1 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, food-borne botulism is the main form of the disease whereas only a few cases of infant botulism have been identified [5].

The BoNTs are divided into seven toxinotypes (A to G) according to their immunological properties and into 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

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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 previously isolated *C. botulinum* strains 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 no. 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 a DNA stool kit (Qiagen, Hilden, Germany) 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 *Bam*HI and *Sal*I sites (underlined), and cloned into pET28a. The resulting plasmid introduced into *Escherichia coli* BL21(DE3) was verified by DNA sequencing. The *C. botulinum* penicillinase (CBP) with an N-terminal 6-His tag was produced and purified as described elsewhere [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]. Ten-fold serial dilutions were made of samples in 50 mM phosphate buffer (pH 6.5) containing 1% gelatin and 0.5 mL was injected intraperitoneally into Swiss mice weighing 20–22 g (Charles River Laboratories, L'Arbresle, France).

Whole genome sequencing

Whole genome sequencing libraries performed using the NEBNext Ultra DNA Library Prep kit for Illumina (New England Biolabs, Ipswich, MA, USA) were sequenced on MiSeq or HiSeq2000 machines (Illumina, San Diego, CA, USA). Sequence files were generated using Illumina Analysis Pipeline version 1.8 (CASAVA; Illumina). After quality filtering, reads were assembled using CLC software version 4 (CLC Bio LLC, Cambridge, MA, USA).

Results

Case report

On 21 February 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 MRI were normal. Three-Hertz 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 findings but a muscle biopsy was normal. The symptoms persisted and a second electromyogram 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 because of the descendant 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 (Fig. 1 and see Supplementary material, Table S1). The baby received amoxicillin (50 mg/kg, three times per day intravenously) and metronidazole (40 mg/kg in three intravenous administrations per day) for 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, Novartis Vaccines and Diagnostics, Marburg, Germany) associated with amoxicillin and metronidazole at the same posology for 8 days by intravenous route and two additional days by oral route. A rapid improvement was observed 2 days after the anti-toxin and antibiotic administration. The baby was fed with breast milk and received probiotics (Biogaïa® 5 drops/day, Lactéol® 2 bulbs/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 three oral administrations per day) for 10 days, and recovered gradually from 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 Fig. 1 and in the Supplementary material (Table S1). 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. *Clostridium botulinum* A was found in stools up to 114 days after the onset of the symptoms (Fig. 1, and see Supplementary material, Table S1).

Antibiotic susceptibility of C. botulinum strains

The six *C. botulinum* strains isolated from stool samples (Fig. 1, and see Supplementary material, Table S1) 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 on the multi-locus sequence typing 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 mg/L) to penicillins and to cephalothin. Moreover, inducible, heterogeneous and reversible resistance to metronidazole was observed in strain 224-13, as already described for *Clostridium difficile* [18]. Colonies grew inside the inhibition zone after 48 h of incubation with MICs ranging from 1 to >256 mg/L. It is noteworthy that albeit the agar disc diffusion method is not recommended by the CLSI, the diffusion method with metronidazole disc (5-μg disc) allowed the detection of