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Original article

Molecular diversity of *Clostridium botulinum* and phenotypically similar strains

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Abstract

This study was undertaken to examine phenotypic and genetic features of strains preliminary classified as *Clostridium botulinum* species. The phenotypic characteristics were assessed with different culture media and biochemical tests. The genetic characterization included detection of botulinum toxin genes by PCR and macrorestriction analysis with *Sma*I, *Xho*I and *Sac*II by PFGE (Pulsed-field Gel Electrophoresis). Despite similar biochemical properties of all analysed strains, only 47% of them contained genes determining toxicity specific to *C. botulinum* species. The most valuable differentiation of *C. botulinum* and *C. botulinum*-like strains was obtained after *Sma*I digestion. The highest affinity was observed among *C. botulinum* type B profiles which was even up to 100%. It was found 100% of affinity between *C. botulinum* and *C. botulinum*-like strains, however, the similarity among *C. botulinum* and *C. botulinum*-like was generally lower than 80%.

Key words: *Clostridium botulinum*, biochemical properties, PCR, PFGE

Introduction

Clostridium botulinum is a Gram-positive, spore-forming, rod-shaped anaerobic bacterium, widely distributed in soil, sediments of lakes and decaying vegetation. The anaerobe produces the most potent toxins in the environment, botulinum toxins (BoNTs). Eight serotypes (A-H) of *C. botulinum* are recognized on the basis of different antigenic properties of their respective toxins (Dover et al. 2014). *Clostridium botulinum* strains are divided into four distinct metabolic groups (I-IV) based on biochemical properties (Hatheway 1990, 1995, Saeed 2004). The first group consists of proteolytic *C. botulinum* strains able to produce A, B, and F toxins and non-toxigenic *C. sporogenes.* The second group includes A, B, E non – protelytic *C. botulinum* toxotypes. The third group consists of proteolytic and non – proteolytic C, D *C. botulinum* toxotypes and also *C. novyi* type A. Group IV consists of *C. botulinum* type G (also determined as *C. argentinense* in literature), and also *C. hastiforme* and *C. subterminale* (Suen et al. 1988, Hatheway 1990, 1995, Rosseto et al. 2014). These physiological groups were based on saccharolytic and proteolytic features, alcohol fermentation end products, heat resistance of spores, and ability to grow in the presence of acids, salts and alcohol, growth in different temperatures (Hatheway 1990).

The diversity of the neurotoxin-producing clostridia has been supported by molecular taxonomic studies (Collins and East 1998, Hill and Smith 2013). Further 16S rRNA analyses of the Clostridia con-

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firmed the classification of *C. botulinum* into four groupings which corresponded to the four mentioned physiological groups (Collins and East 1998). The experiment on 174 *C. botulinum* strains conducted by using sequencing analysis of 16S rRNA, BoNT genes and by using amplified fragment length polymorphism (AFLP) confirmed the existence of at least four distinct genomic backgrounds, each of which has likely independently acquired BoNT genes through horizontal gene transfer (Collins and East 1998, Hill and Smith 2013). In addition to *C. botulinum* strains, *C. butyricum* and *C. baratii* are known to produce BoNT types E and F, respectively (Hall et al. 1985, Suen et al. 1988). Because the other BoNT-negative Clostridia species are also classified to the above described groups, this classification based on ability to produce botulinum toxins is inaccurate. Moreover, there are no selective media for isolation of all strains classified to *C. botulinum* species which complicate the pathogen identification by traditional culture methods (Gryko et al. 1990, Linstrom and Korkeala 2006). The reference method for BoNTs and *C. botulinum* detection according to the Association of the Official Analytical Chemists (AOAC) is the mouse bioassay (Cunniff 1995). It is based on the intraperitoneal mouse injection of supernatant obtained from suspected culture. At the same time, negative controls are conducted. The occurrence of specific, nervous symptoms in animals confirms the *C. botulinum* presence. The variability of phenotypic features among the strains and high probability of toxin gene lost through passages make the mouse bioassay an insufficient tool for a reliable diagnosis of botulism (Gryko et al. 1990). Furthermore, the examination is also labour- and time-consuming, and ethically controversial (Gryko et al. 1990, Lindström and Korkeala 2006). The molecular methods focused on BoNT genes detection have become a supplementary tool to the mouse lethality assay. They also allow to distinguish *C. botulinum* from *C. botulinum*-like strains (Lindström and Korkeala 2006).

The aim of this study was to examine biochemical and genetic diversity of *C. botulinum* and *C. botulinum*-like strains of various origins collected through the last four decades in Poland.

Materials and Methods

Isolates

A total of 68 bacterial strains preliminary classified as *C. botulinum*-like were studied. Previously, along the collection period, these strains have been classified to *C. botulinum* species. Sixty of them were classified to the I metabolic group and another 8 strains to the II metabolic group. Bacteria were obtained from the Sera and Vaccines Manufacturing (SIS BIOMED) Company in Warsaw ($n=26$) and from the Military Institute of Hygiene and Epidemiology (MIHE) in Warsaw ($n=26$). Moreover, food and feed isolates ($n=12$) phenotypically similar to *C. botulinum* species and *C. botulinum* reference strains from National Collection of Type Cultures, type A (NCTC 887), B (NCTC 3815), E (NCTC 8266) and F (NCTC 10281) were analyzed.

Cultural characterization

The cultural properties of anaerobic bacteria on agar media were evaluated. The strains deposited in Cryobank (Mast Diagnostica, UK) were inoculated on Fastidious Anaerobe Agar and Willis-Hobbs medium. After incubation at 37°C for 48 h in anaerobic conditions, phenotypic properties of analysed strains were evaluated (lipolysis, proteolysis). The lipholytic properties were evaluated by notification of „pearl layer" on the surface of grown colonies which demonstrate the releasing of free fatty acids from the complexes of triacylglycerols from egg yolk (ingredient of the differential media mentioned). The proteolytic abilities were observed as the brightening zones surrounding the colonies obtained on the FAA and Willis-Hobs solid media. The microscopic slides with anaerobe cultures for fresh Gram staining were also prepared.

Mouse bioassay

The Clostridia strains were examined by using lethal mouse bioassay according to the AOAC protocol (Cunniff 1995). In the examination, 136 laboratory mice were used. For the seroneutralization purposes, polyvalent botulinum antitoxin ABEF was used (CDC, USA).

Preparation of DNA for PCR analyses

The DNA was isolated from 3 to 5 characteristic colonies obtained on FAA and Willis-Hobbs surfaces. The extraction process was carried out using commercial set for DNA preparation – Genomic Mini A&A Biotechnology (Gdynia, Poland) strictly according to protocol supplied by producer.

Molecular characterization by PCR

For *C. botulinum ntnh* gene detection, the set of seven primers and TaqMan probe with locked nucleic acids (LNA) bases were used according to Raphael and Andreadis (2007). The description of sequences are included in Table 1. The above set facilitated the amplification of a highly conserved region common for all seven toxotypes occurring in *C. botulinum* species and encoding nonhemagglutinin component of BoNTs (NTNH) (Raffestin et al. 2004, Raphael and Andreadis 2007). The reaction mixture contained 5 µl of DNA template, 4 µl of LightCycler TaqMan Master (Roche), 0.7 µM of each primer, and 0.24 µM of NTNH410 probe. The real-time PCR was performed on the LightCycler 2.0 System (Roche). Following 10 min activation step at 95°C, reactions were subjected to 40 cycles, consisting of three steps: at 95°C for 15 s, at 42°C for 15 s, and at 55°C for 60 s. Fluorescence data were acquired following the third step of each cycle (55°C).

For identification of *C. botulinum* toxotype A, B, E or F, the set of eight primers was used, according to De Medici et al. (2009). The description of sequences are included in Table 2. It enabled detection of genes encoding particular BoNTs in toxotype A, B, E and F. The reaction mixture contained 5 µl of DNA template, 2.5 µl of 10xTaq buffer with KCl (Fermentas), 0.3 µM of each primer, 4 mM of $MgCl_2$ (Fermentas), 200 µM of dNTP mixture (Fermentas), and 1.25U of Taq DNA polymerase (Fermentas) and the final volume of reaction mixture was 25 µl. The mPCR was performed on T1 Thermocycler (Biometra). Following initial denaturation step at 95°C for 60 s, reaction was subjected to 27 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 3 min. The final extension was carried out at 72°C for 3 min. The mPCR products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide, located in 1xTBE buffer (Fermentas) for 1.5 h under 100 V. Ten µl of reaction mixture and 2 µl of loading buffer 6xDNA Loading Dye (Fermentas) were loaded into each well. The molecular weight of the obtained products were determined on the basis of a molecular weight marker – GeneRuler 100bp DNA Ladder Mix (Fermentas). After separation, PCR products were analysed under the UV light transluminator (Vilber-Lourmat).

Biochemical characterization

The biochemical features of analysed strains were estimated by Rapid ID 32A and API 20A assays (bioMérieux). The Rapid ID 32A and API 20A test strips were read visually and by the autoreader, mini API (bioMérieux), and its database version 1.3.1. was used. All steps of preparation the biochemical tests were conducted strictly according to descriptions included in protocols supplied by the producer of tests (bioMérieux).

Molecular subtyping by PFGE

All *C. botulinum* and *C. botulinum*-like isolates were typed by PFGE protocol developed according to the procedures described by Hielm et al. (1998), Nevas et al. (2005) and PulseNet PFGE Manual (CDC 2004). Briefly, the strains were cultured in test tubes with tryptone-peptone-glucose-yeast extract broth (TPGY) during 48 h at 37°C, under anaerobic conditions. Subsequently, two drops of the culture from the bottom of the probe were spread on the Columbia agar (pancreatic digest of casein 12.0 g, peptic digest of animal tissue 5.0 g, yeast extract 3.5 g, beef extract 3.0 g, corn starch 1.0 g, sodium chloride 5.0 g, agar 13.5 g) with 5% of sheep blood and incubated at 37°C for 24 h, under anaerobic conditions. Afterwards, the culture obtained was suspended in 2 ml of PIV buffer (1M NaCl, 10 mM Tris-HCl, pH 7.6) with 4% formaldehyde. Bacterial suspension was then prepared with a density of 7.5 McF with Densimat (BioMerieux). The suspension was centrifuged at 1260 x *g* for 10 min and the supernatant was decanted. The pellet obtained was resuspended in PIV buffer with 4% of formaldehyde and centrifuged once more. After the second centrifugation the pellet was suspended in 2 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Subsequently, 60 µl of 0.1% lysozyme (Oxoid) was added to 240 µl of the suspension, and subjected to thermolysis in Thermomixer comfort (Eppendorf) at 37°C for 10 min. To 300 µl of lysate at 50°C was added 1.2% low melting point agarose (Prona), 1% sodium dodecyl sulfate (Oxoid) and 0.2 mg proteinase K (Fermentas), and the mixture was poured into plug molds. After solidification at 4°C, the plugs were transferred into 50 ml tubes containing 4 ml Lysis buffer (50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1% L-laurylosarcosine sodium salt) and then placed in 37°C water bath for 2 h with gentle shaking. Afterwards, the lysis buffer was removed and plugs were washed twice in redistilled water in 50°C water bath for 10 min with gentle shaking. Then, washings of the plugs were done four times with TE buffer at 50°C for 15 min. Then, the plugs were stored in TE buffer at 2-8°C or directly digested with restriction enzymes. Three restriction enzymes for *C. botulinum* DNA digestion, *Sma*I (CCC↓GGG), *Xho*I (C↓TCGAG) and *Sac*II (CCGC↓GG) (Fermentas) were used. The *Sma*I and *Xho*I concentration was

2U/µl and SacII 4U/µl, and the plugs were digested for 4h

at 37°C. The restriction fragments of DNA were separated in 1% agarose in 0.5X TBE buffer using contour-clamped homogenous electric field (CHEF-DRIII, Bio-Rad). The electrophoresis was conducted for 21 h at 14°C, 6V/cm, with initial and final switching times of 0.4 s and 40 s. DNA of *Salmonella* serotype Braenderup strain H9812 was digested with *Xba*I (T↓CTAGA) and used as molecular weight standard (CDC 2004). Strain H9812 was provided by the National Reference Laboratory for *Salmonella* at the National Veterinary Research Institute in Pulawy (Poland). Band-based dendrograms were produced by using an unweighted pair-group method using arithmetic averages (UPGMA) with Dice correlation coefficient and 2% tolerance of band position. The PFGE gels were analysed using the fingerprint analysis software (BioNumerics, Applied Maths, Belgium).

Results

Cultural characterization

A total of 68 strains (100%) were irregular, cream to pale yellow and gently protuberant on Willis-Hobbs agar. They had a mother-of-pearl layer due to the action of lipase on the fat in the medium. All analysed strains were also surrounded by zone of precipitate. The proteolytic properties exhibited 60 strains (88%) and it was visible through the zone of illumination around colonies. On FAA agar also irregular, grey to cream-yellow and gently protuberant colonies were observed. Colonies of all *C. botulinum* strains were covered and surrounded by a thin, pearl layer due to lipase production and lecithinase was also formed by all the strains. The proteolytic properties were difficult to confirm because of pale colour of medium. Microscopic characters revealed Gram-positive, non-motile rods.

Mouse bioassay

The results of mouse bioassay shown that only 32 (47%) of examined strains were able to produce BoNTs. The occurrence of specific, nervous symptoms was observed in mice such as labored abdominal breathing, wasp shape, ruffled fur, weakness of the limbs progressing to total paralysis, gasping for breath (opening of the lower jaw) and death due to respiratory failure.

Molecular characterization by PCR

Among all 68 strains phenotypically similar to *C. botulinum* species, only 32 strains (47%) possessed *ntnh* and *bont* genes, which determine BoNT production. The results were compatible with those of mouse bioassay. Six strains (28%) were classified as type A, 23 strains as type B (72%), 2 strains as type AB (6%) and the single strain was type F (3%).

Biochemical characterization

According to the Mini API system database, most of *C. botulinum* 18 (56%) and *C. botulinum*-like 22 (61%) strains examined by Rapid ID 32A were classified to *C. sporogenes* species. The percent of identity ranged from 89.4 to 99.9%. Only 4 (13%) of *C. botulinum* strains and 4 (12%) *C. botulinum*-like strains examined were classified to *C. botulinum* group I species with percent of identity ranging from 84.2% to 98.1%. Our study demonstrated that 16 (50%) of *C. botulinum* isolates were α-glucosidase--positive and 18 (56%) produced β-glucosidase. Among *C. botulinum*-like strains, 22 (61%) were α-glucosidase-positive whilst 17 (48%) were able to produce β-glucosidase. α-Glucosidase and β-glucosidase production according to the Mini API system are considered characteristic features for *C. sporogenes* species. We also observed strain of *C. botulinum* type AB that did not show biochemical characteristics typical of the species according to the Bergey's Manual of Systematic Bacteriology (Cato et al. 1986). It was a single strain type AB, which did not ferment glucose, despite the repeated testing by API 20A. Only one of the strains were was classified to the *C. botulinum* group II species. In case of the other strains examined by using Rapid ID 32A tests, the Mini API system classification was not acceptable.

The results of API 20A tests were interpreted by using Mini API System. The interpretation shown that 29 (90%) of *C. botulinum* and 31 (85%) of *C. botulinum*-like strains examined were classified to *C. botulinum*/*C. sporogenes* species. The identity in this test was estimated at 99%. The identification of the other strains was were not acceptable. This system is invalid in the case of identification of *C. botulinum* group II strains.

Molecular subtyping by PFGE

A total of 68 isolates were submitted to macrorestriction analysis by PFGE. After *Sma*I digestion 60 DNA patterns were noted, where 30 strains belonged

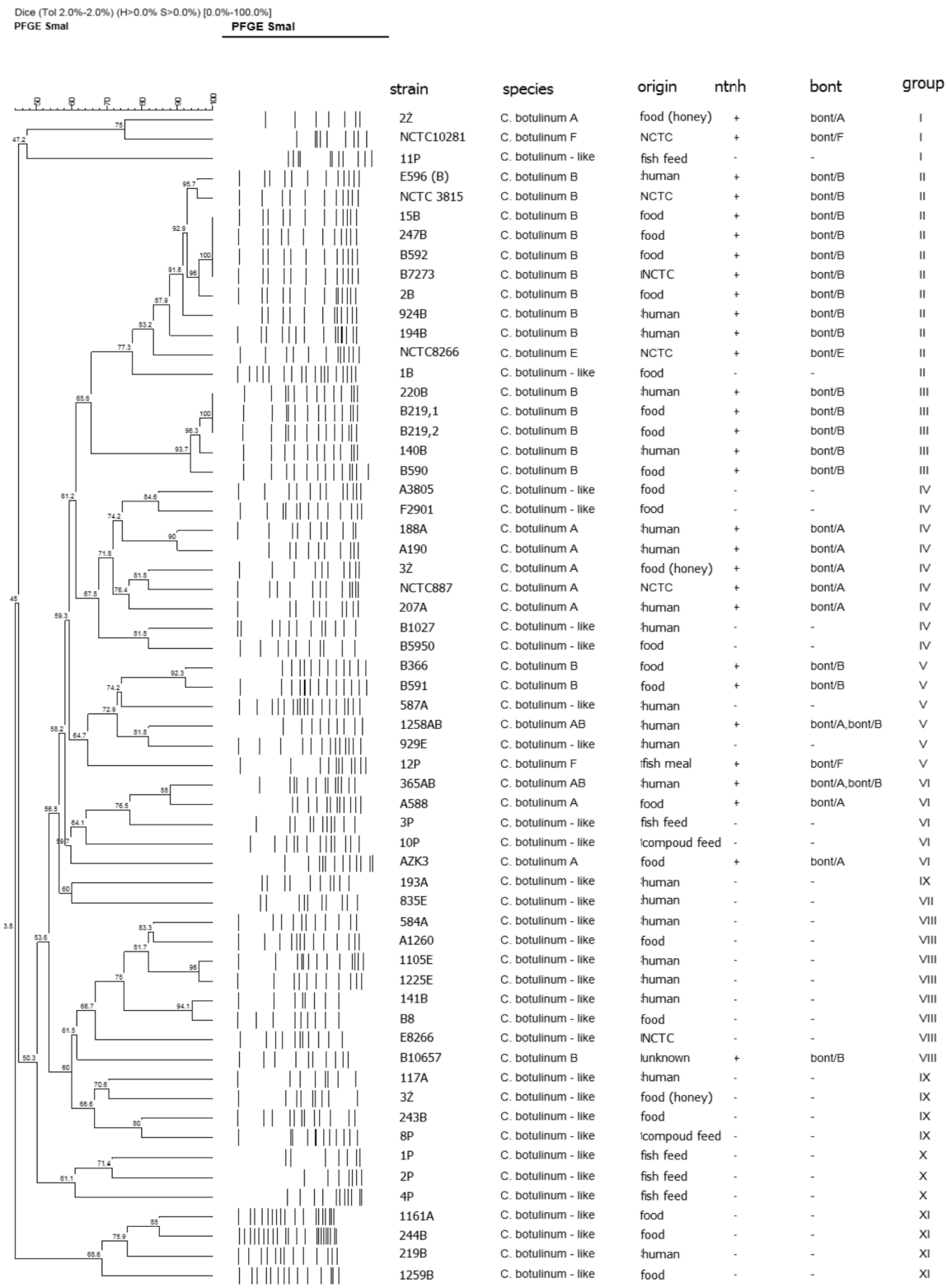
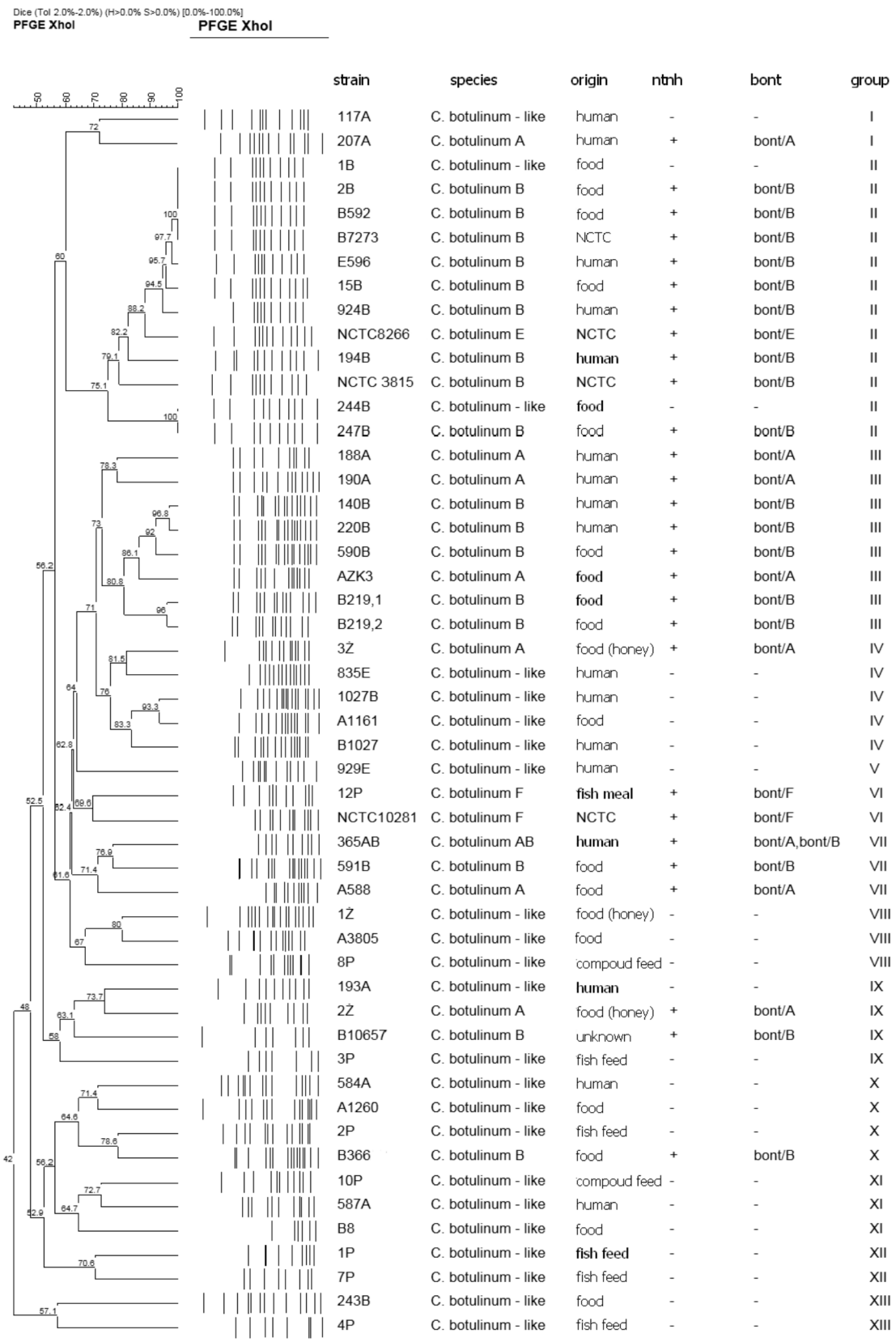


Fig. 1. *SmaI*-PFGE dendrogram based on the Dice coefficient indicating the genetic relatedness of the 60 *C. botulinum* and *C. botulinum*-like isolates. The scales at the top indicate the similarity indices (in percentage).



strain	species	origin	ntnh	bont	group
117A	C. botulinum - like	human	-	-	I
207A	C. botulinum A	human	+	bont/A	I
1B	C. botulinum - like	food	-	-	II
2B	C. botulinum B	food	+	bont/B	II
B592	C. botulinum B	food	+	bont/B	II
B7273	C. botulinum B	NCTC	+	bont/B	II
E596	C. botulinum B	human	+	bont/B	II
15B	C. botulinum B	food	+	bont/B	II
924B	C. botulinum B	human	+	bont/B	II
NCTC8266	C. botulinum E	NCTC	+	bont/E	II
194B	C. botulinum B	human	+	bont/B	II
NCTC 3815	C. botulinum B	NCTC	+	bont/B	II
244B	C. botulinum - like	food	-	-	II
247B	C. botulinum B	food	+	bont/B	II
188A	C. botulinum A	human	+	bont/A	III
190A	C. botulinum A	human	+	bont/A	III
140B	C. botulinum B	human	+	bont/B	III
220B	C. botulinum B	human	+	bont/B	III
590B	C. botulinum B	food	+	bont/B	III
AZK3	C. botulinum A	food	+	bont/A	III
B219,1	C. botulinum B	food	+	bont/B	III
B219,2	C. botulinum B	food	+	bont/B	III
3Ż	C. botulinum A	food (honey)	+	bont/A	IV
835E	C. botulinum - like	human	-	-	IV
1027B	C. botulinum - like	human	-	-	IV
A1161	C. botulinum - like	food	-	-	IV
B1027	C. botulinum - like	human	-	-	IV
929E	C. botulinum - like	human	-	-	V
12P	C. botulinum F	fish meal	+	bont/F	VI
NCTC10281	C. botulinum F	NCTC	+	bont/F	VI
365AB	C. botulinum AB	human	+	bont/A,bont/B	VII
591B	C. botulinum B	food	+	bont/B	VII
A588	C. botulinum A	food	+	bont/A	VII
1Ż	C. botulinum - like	food (honey)	-	-	VIII
A3805	C. botulinum - like	food	-	-	VIII
8P	C. botulinum - like	compoud feed	-	-	VIII
193A	C. botulinum - like	human	-	-	IX
2Ż	C. botulinum A	food (honey)	+	bont/A	IX
B10657	C. botulinum B	unknown	+	bont/B	IX
3P	C. botulinum - like	fish feed	-	-	IX
584A	C. botulinum - like	human	-	-	X
A1260	C. botulinum - like	food	-	-	X
2P	C. botulinum - like	fish feed	-	-	X
B366	C. botulinum B	food	+	bont/B	X
10P	C. botulinum - like	compoud feed	-	-	XI
587A	C. botulinum - like	human	-	-	XI
B8	C. botulinum - like	food	-	-	XI
1P	C. botulinum - like	fish feed	-	-	XII
7P	C. botulinum - like	fish feed	-	-	XII
243B	C. botulinum - like	food	-	-	XIII
4P	C. botulinum - like	fish feed	-	-	XIII

Fig. 2. *Xho*I-PFGE dendrogram indicating the genetic relatedness of the 51 *C. botulinum* and *C. botulinum*-like isolates.

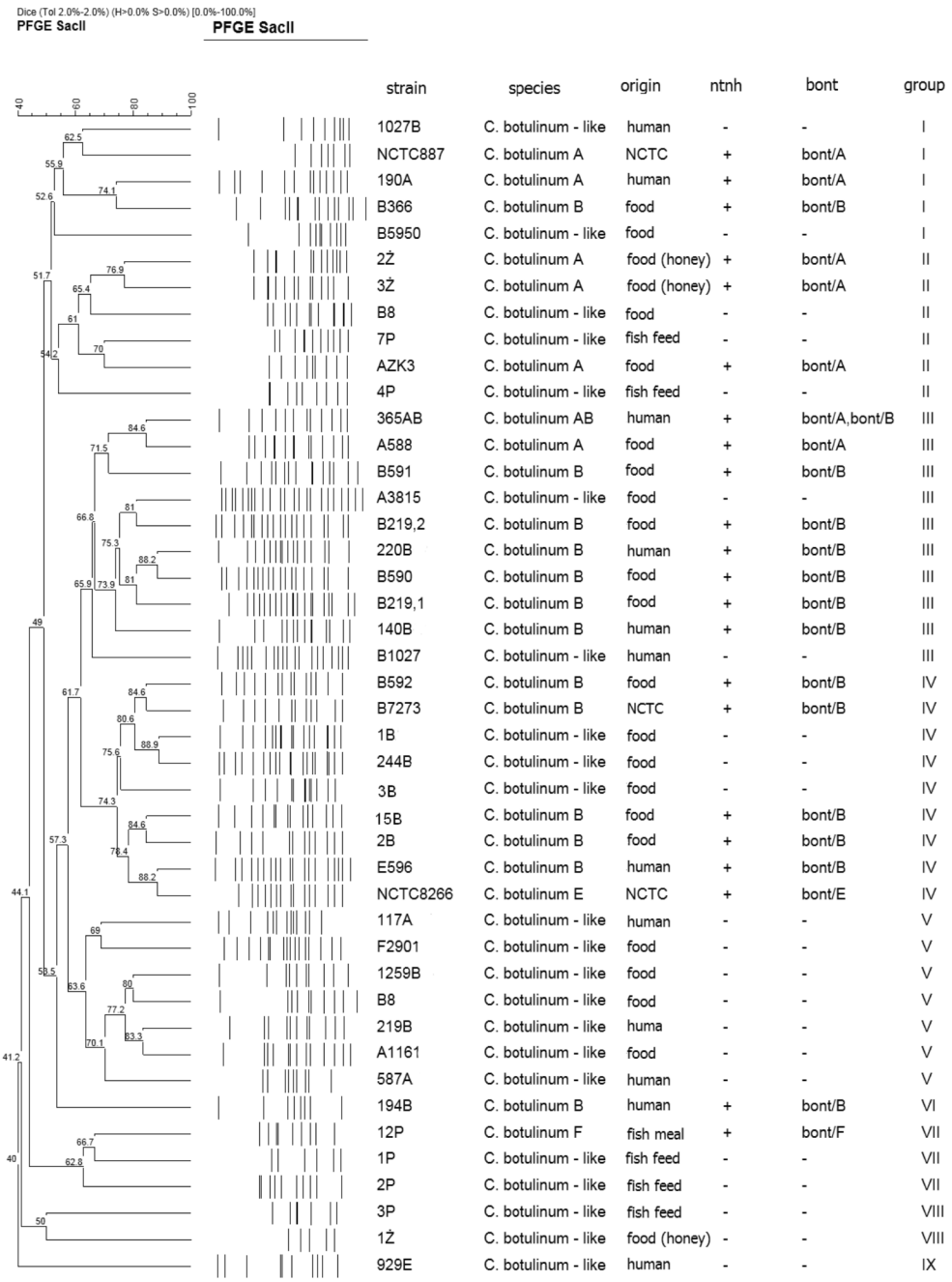


Fig. 3. *Sac*II-PFGE dendrogram indicating the genetic relatedness of the 44 *C. botulinum* and *C. botulinum*-like isolates.

to *C. botulinum* and the next 30 isolates were *C. botulinum*-like strains. Clonal relationship among the strains ranged from 44% to 100%. The restriction patterns had between 7 and 22 bands with approximate sizes of 33 to 1135 kb. However, these 60 isolates could be divided into 11 groups (I – XI) containing between 1 and 11 isolates per group, according to the Dice coefficient of similarity (Fig. 1). The major genetic relatedness was observed in II and III group containing 4 and 3 isolates, respectively, with indistinguishable PFGE patterns (100% similarity). The affinity between *C. botulinum* and *C. botulinum*-like strains did not exceed 81.8%.

The *Xho*I-PFGE profiles (n=51) could be divided into 13 groups (I-XIII) containing between 1 and 12 isolates per group and restriction patterns had between 6 and 17 bands (Fig. 2). There were 28 *C. botulinum* and 23 *C. botulinum*-like patterns. The majority of patterns was represented by one isolate. This group also revealed genetically identical (100% similarity) isolates (n=6). It is worth mentioning that, PCR-based identification classified them as *C.*

botulinum type B ($n=4$) as well as *C. botulinum*-like ($n=2$). Clonal relationship in the *Xho*I-PFGE profiles ranged from 42% to 100% and the similarity between *C. botulinum* and *C. botulinum*-like strains ranged from 58% to 100%. The most divergent PFGE patterns were in XIII group where similarity amounted to 57.1%.

Based on the size and number of bands, 44 *Sac*II-PFGE patterns and 9 groups (from 1 to 10 isolates) were distinguished, among them 22 isolates were *C. botulinum* and 22 *C. botulinum*-like. The profiles consisted of 5 to 23 restriction fragments and molecular mass also ranged from 33 to 1135 kb (Fig 3). There were no genetically identical isolates. Clonal relationship ranged from 40% to 90% and it was significantly lower than *Sma*I and *Xho*I patterns. The similarity between *C. botulinum* and *C. botulinum*-like strains did not exceed 76%. The highest similarity was observed between two *C. botulinum*-like strains and it was 90%. The most divergent PFGE patterns were that of VIII group and the identity was at 50%.

Discussion

PCR-based methods to overcome difficulties connected with *C. botulinum* identification were used. Only about half of the strains suspected to be *C. botulinum*, revealed the genes specific to the species, despite phenotypic similarities. Among the analyzed *C. botulinum* isolates type B strains prevailed. Unfortunately, unknown is the exact number of *C. botulinum* strains, that lost their toxicity genes in the course of laboratory isolation, because of panmictic nature of *C. botulinum* species as well as another *Clostridium* species (Zhou et al. 1993, Johnson and Bradshaw 2001, Sawires and Songer 2006, Bradshaw et al. 2010, Wangroonsarb et al. 2014, McCallum et al. 2015).

The comparison between *C. botulinum* and *C. botulinum*-like strains shown their high biochemical similarity. The common features for the highest number of analyzed strains were glucose and maltose fermentation, and α-glucosidase, β-glucosidase production. The biochemical properties are not sufficient to distinguish *C. botulinum*-like from *C. botulinum*, which was confirmed by other authors. Lindstrom et al. (1999) observed that API 20A test was not able to distinguish between proteolytic *C. botulinum* and *C. sporogenes*. α-Glucosidase- and β-glucosidase-positive *C. botulinum* strains were classified as *C. sporogenes*. The highest efficiency and the most satisfactory effect of macrorestriction PFGE analysis were obtained by using *Sma*I enzyme. The similar results were obtained by other authors (Hielm et al. 1998, Nevas et al. 2005, Umeda et al. 2013). Hielm et al. (1998) among 15 restriction enzymes used to the analysis of *C. botulinum* strains obtained from marine environment, recommended *Sma*I and *Xho*I. Nevas et al. (2005) recommended using of *Sma*I enzyme, because differentiation of F toxin type was possible in the opposition to the usage of *Xho*I and *Sac*II.

The PFGE revealed heterogeneity of the analysed *C. botulinum* isolates, however also a high similarity level between the species and *C. botulinum*-like isolates was observed. According to *Xho*I-PFGE dendrogram, *C. botulinum*-like 1B and 244B from group II were identical to type B *C. botulinum* 2B, B592, B7273 and 247B, respectively. High relatedness of some analysed strains confirms the fact that the same isolates ($n=11$) were included in group II by *Sma*I- as well as *Xho*I-PFGE, and 8 of them were classified together in group IV after *Sac*II restriction. It is interesting that among closely related strains of *C. botulinum* type B there was also one strain of type E, which confirms panmictic nature of the anaerobe. The high similarity between *C. botulinum* and *C. botulinum*-like strains was also observed by Carlier et al. (2004) taking into account 16S rDNA sequence. The similarity between *C. botulinum* type B (ATCC 25765) and *C. botulinum*-like strain AIP 355.02 reached the level of 93%. The high percentage of similarity between *C. botulinum* and *C. botulinum*-like strains was also mentioned by Nakamura et al. (1977), Lee and Riemann (1970). They found that homology between this kind of strain sequences was more than 70%.

The isolates numbered 219B, B219.1 and B219.2 as well as 220B originated from the same human botulism case and all these strains initially produced BoNT B. *Clostridium botulinum*-like 219B strain lost genes determining toxicity during passages resulting in distant relatedness with the above mentioned isolates. This deletion decreased isolate relatedness from 100% to 40% by *Sma*I-PFGE and from 81% to 60% according to *Sac*II-PFGE. The 100% relatedness of isolates from single botulism case and the sharp decline of relatedness by *Sma*I-PFGE confirms a higher utility of *Sma*I enzyme in *C. botulinum* macrorestriction analysis. Moreover, digestion of genomic DNA with *Sma*I resulted in the highest number of readable profiles ($n=60$) in comparison to the other enzymes, and obtained profiles revealed above 10 restriction fragments.

The obtained similarity levels of *C. botulinum* profiles indicated the differences among particular toxin types. In most cases, A and B toxin types were compared. In the case of type B strains, the similarity achieved the level of 100% after digestion with *Sma*I and *Xho*I, whilst after using of *Sac*II enzyme this level

was available at 88%. After using *Sma*I, the similarity between A and B profiles did not exceed 74%, whilst by using *Xho*I 72% and *Sac*II-74%. The differences among particular profiles were observed by Nevas et al. (2005). For the differentiation of *C. botulinum* strains, they recommended *Sac*II enzyme or its combination with *Sma*I and *Xho*I. After digestion of genomic DNA by *Sac*II, they obtained A and B types *C. botulinum* profiles, the similarity of which did not exceed 80%, whilst among type A strains profiles and type B the similarity reached 100%.

The ability to produce BoNTs by *C. botulinum* strains demands a suitable training for the laboratory staff handling the pathogens from III risk group (Johnson et al. 2005). The *C. botulinum*-like strains, which show high biochemical and genetic similarity to *C. botulinum* could be used for laboratory staff improving trainings purposes within this pathogen identification. Such trainings were recommended by Bradshaw et al. (2010) who conducted the research on non-toxinogenic mutant of *C. botulinum* 62A.

Until now we have not disposed any selective media for growth inhibition of the other anaerobic microflora which compete with *C. botulinum* for nutritional ingredients. Isolation process of this microorganism could be limited by occurrence of the other species with smaller culturing demands *e.g. C. perfingens* or *C. tetani.* (Smith 1975, Lindstrom and Korkeala 2006, Kukier and Kwiatek 2010). According to Kukier and Kwiatek (2010) the percentage of feeds contaminated by *C. perfringens* may reach 68% and 4% of food of animal origin and occurrence of this microorganism could be inhibitory for the *C. botulinum* growth. The isolation process of this pathogen is also inhibited by the occurrence of anaerobic *C. botulinum*-like bacteria which have not the ability to produce BoNTs. In the literature, toxigenicity loss among A, B, E and F toxin types has been described (Zhou et al. 1993, Johnson and Bradshaw 2001, Bradshaw et al. 2010). The classification of suspected microorganism to *C. botulinum* species, based only on biochemical characterization, is not reliable. The PCR and real-time PCR methods described in this study could be used as an excellent tool in *C. botulinum* detection and determination of toxin types of this pathogen, as it was proved by compatible results of MBA test. The most effective genotyping was achieved with the use of SmaI, after treating genomic DNA with this enzyme the PFGE profiles obtained enabled sharp distinguishing between *C. botulinum* and *C. botulinum*-like strains and also between particular toxin types of this pathogen.

As shown above, phenotypic characterization of strain suspected of *C. botulinum* is not sufficient for a reliable identification of this species and needs genetic characterization or the other tests for determining toxigenicity of the suspected strain. According to the authors opinion, the future classification should be based on the expression of genes which determine toxigenicity of suspected strains.

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