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Review

Methods and difficulties in detection of *Clostridium botulinum* and its toxins

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Abstract

The aim of this work was to present selected data regarding traditional and modern methods for *C. botulinum* and its toxins detection. In this article, methods based on culturing techniques, mouse bioassay, immunological techniques, chromatography and PCR, PFGE, RFLP, AFLP are described. The mentioned techniques were evaluated considering their usefulness in the samples examination, genotyping of strains and the diagnostics of botulism.

Key words: *Clostridium botulinum*, BoNTs, molecular biology, genotyping.

Introduction

Botulism has been affecting human civilization from the earliest time. However, the first, accurately described incident of a food-borne botulism was documented as late as the 18th century, when the consumption of meat and blood sausages was a cause of many deaths in the Kingdom of Württemberg, in the South Western Germany (Erbguth 2008). Before this time, the botulism among humans and animals had not been clearly associated with the consumption of food or feed. The district medical doctor Justinus Kerner has published the first accurate and complete descriptions of the symptoms of food-borne botulism (Kerner 1820). *Clostridium botulinum* was isolated for the first time in 1895, when Belgian scientist Emile Pierre van Ermengem had considered human intoxication outbreak after the consumption of salted ham. The intoxication outbreak and the scientist's experiments on isolated bacterium named *Bacillus botulinus*, were described in 1897 (van Ermengem 1897). At the beginning of 20th century, the name of bacterium was changed into *Clostridium botulinum* (Erbguth 2008). Chronology of discovered toxotypes of this bacterium is indicated by subsequent letters of alphabet from A to G. Toxotype discovered by van Ermengem was marked as A and the last as G (Ciccarelli and Gimenez 1972). After 1970s a definition stating that all microorganisms which are able to produce botulinum toxins are classified to *C. botulinum* species was formulated (Cato et al. 1986). Nowadays, this definition does not apply. According to the history of discovering subsequent *C. botulinum* toxotypes it is worth to ask – why botulism cases were not always associated with the occurrence of this microorganism. The answer can be found in the further findings. In 1985, Hall et al. (1985) found a strain of *C. barati* that produced type F botulinum toxin. Aureli et al. (1986) isolated *C. butyricum* strains that produced toxin type E. There had been also observed similar biochemical features between *C. botulinum* and the other species of *Clostridia* which have no ability of botulinum toxins

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production. Furthermore, a transfer of genes among A, B, E, F toxotypes was noticed and also that the toxigenicity of *C. botulinum* type C and D is determined by lisogenic process of specific bacteriophages. The problems mentioned above created a base of classification defined in 1990s according to which there are four metabolic groups of *C. botulinum*, and the *C. botulinum – like* strains (with similar phenotypic features, which have no ability of botulinum toxins production). It was observed that among strains which are able to produce botulinum toxins, high biochemical and genetic diversity exists. 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 includes *C. botulinum* type G (with regard to genetic and phenotypic differences between the other *C. botulinum* strains, also determined as *C. argentinense* in the literature), and also *C. subterminale* and *C. hastiforme* (Suen et al. 1988, Hatheway 1990, Hatheway 1995, Hyytiä-Trees 1999). Phenotypically, group I and II strains differ from each other significantly. Group I and III organisms seem to be more of terrestrial origin and are present in temperate climates, whereas group II strains, particularly type E, are frequently found in aquatic environments in the northern hemisphere. Differences in spore heat resistance and growth temperatures are responsible for the risks posed by *C. botulinum* groups I , II and III in the food and feed industry. Group I spores, which have a high heat resistance (Ito et al. 1967, Lynt et al. 1981), cause problems in canning and home preservation of vegetables and meat, whereas group II spores, with somewhat lower spore heat resistance (Lindström et al. 2003), are of a great concern in low processed packaged foods that have extended shelf lives at refrigerated temperatures (Peck et al. 1997). Group III is considered as a major causative botulism agent in animals and toxigenicity of these microorganisms is determined by specific bacteriophages (Saeed 2004).

Clostridium botulinum isolation

The diversity among *C. botulinum* strains and occurrence of *C. botulinum*-like strains cause problems with an isolation process. The growth of this species requires obligatory anaerobiosis. Different nutritional requirements for particular metabolic groups determine usage of proper culture method (Saeed 2004). The main factors which have influence on the *C. botulinum* isolation from examined sample are: incubation temperature, pH value, content of preservatives and presence of competitive microflora. The optimal redox potential for *C. botulinum* growth equal -350 mV, however BoNT production was also observed at +250 mV (Anon 1998). The pH value should be higher than 6.0. The enrichment stage of culturing process is conducted by using broths containing: meat extract, amino acids, various combinations of tryptone, peptone, glucose, yeast extract and ingredients decreasing redox potential like thioglycolates, sodium and potassium sulfide, L-cysteine or liver in pieces (Erbguth 2008). The most popular are enrichment broths, like: Wrzosek broth, Robertson broth, Tryptone Peptone Glucose Yeast Extract Broth (TPGY), Clostridial Medium (CM), Cooked Meat Medium (CMM), Reinforced Clostridial Medium (RCM), Fastidious Anaerobic Broth (FAB) (Saeed 2004, Lindstrom and Korkeala 2006). Before sampling, chosen broth is deoxygenated by heating at 100°C for 10-15 minutes. After sampling into two test tubes with broth, one of them is pasteurized for vegetative cells inactivation and stimulation of spore germination. Because of different resistance to pasteurization temperatures, among spores from particular metabolic groups, it is recommended to heat at 70°C for 15 min. The vegetative cells can be also inactivated by ethanol treatment (Koransky et al. 1978). There are known different solid media for *C. botulinum* isolation, however, their essential ingredient is egg yolk emulsion, which enable demonstration of lipolytic and lecithinolytic properties of strain by creating characteristic "pearl layer". The frequently used agar media are: Willis – Hobbs Agar, Fastidious Anaerobic Agar (FAA), *Clostridium botulinum* Isolation Agar (CBI) and Egg Yolk Agar (EYA) (Glasby and Hatheway 1985, Lindström and Korkeala 2006).

The optimal growth temperature for all *C. botulinum* strains is not established, because of different demands of strains from particular metabolic groups. Regarding this fact, for the group I the optimal temperature ranges from 35°C to 40°C, for group II is established at about 25°C, for group III at about 40°C and for group IV circa 37°C (Saeed 2004). Some literature data revealed that the *C. botulinum* isolation could be possible also without enrichment stage and in this case – sample suspension is inoculated directly onto solid media. Such procedures were applied with CBI and EYA media for infant botulism diagnosis (Glasby and Hatheway 1985). However, taking into account presumptive contamination level of natural samples by this pathogen, which is most often low, sample enrichment is recommended (Dezfulian et al. 1981). The *C. botulinum* growth can be inhibited by

competitive microflora, *e.g.* bacteria from genus *Clostridium, Bacillus* or *Streptococcus.*

Clostridium botulinum and botulinum neurotoxins detection

The extreme potency of botulinum neurotoxin demands rigid requirements to ensure the safety of laboratory workers. Despite the potency of its neurotoxin, the non-invasive and non-contagious *C. botulinum* has been graded as a class II pathogen. The appropriate biosafety level 2 containment facilities and trained personnel are therefore a minimum requirement while dealing with *C. botulinum*. Additional contingencies should be considered whenever aerosol or droplet formation from toxic materials is expected. The definition of additional contingency measures should be based on a risk assessment of activities in each laboratory dealing with *C. botulinum* and its toxin. The efficacy of the pentavalent toxoid formerly used to immunize laboratory staff worldwide has been shown to be lower than expected. Therefore, a careful attention must be paid to safety of the laboratory staff (Lindström and Korkeala 2006).

Generally, detection of *C. botulinum* is based on proving the ability of suspicious strains to produce botulinum toxins (BoNTs). The highest number of methods is based on biological and immunological techniques for botulinum toxins detection following culturing of suspicious strains. In the latest years, chromatographic and molecular biology methods became much more popular.

Biological tests

Currently, mouse lethality assay is the reference method approved by the Association of Official Analytical Chemists (AOAC) for BoNTs detection in botulism investigation (Cunniff 1995). The biological test is based on intraperitoneal mouse injection by supernatant obtained after broth culture centrifugation of strain suspected of belonging to *C. botulinum* species (*C. botulinum*-like strain) or sample extract and the observation of the symptoms. Concurrently, negative controls are conducted which enhance the assay specificity. At first, the antitoxin is intraperitonealy injected to a mouse, half an hour before a tested sample administration. The second negative control depends on thermal inactivation (generally, 80ºC for 10 min) of BoNT assumed present in a sample. BoNT presence in the sample is confirmed when botulism symptoms occurred in mouse after tested supernatant injection and when animals used for negative controls are healthy. Death of mouse in a absence of neurological symptoms is not acceptable, because it may be caused by other microorganisms or chemicals present in the sample or by injection trauma (Wheeler et al. 2009).

Typical botulism symptoms sequence in mouse include ruffled fur, labored abdominal breathing, wasp shape, weakness of the limbs progressing to total paralysis, gasping for breath (opening of lower jaw) and/or death due to respiratory failure. The lethal dose of BoNT for 50% of tested mice (LD_{50}) ranges from 5 pg to 10 pg, whilst the limit of detection BoNT by mouse lethality assay is estimated at 0,01 ng/ml of sample (Smith and Sugiyama 1988). The high sensitivity of biological test is limited in laboratory practice, requiring up to 6 days to obtain final results and is conducted only in facilities that have mouse available for a test. Additional drawbacks of the method include labour- and time-consumption and ethical dubiousness.

Nonlethal mouse assay is used to estimate BoNT/A power (Sesardic et al. 1996). It is based on local, subcutaneous injection of BoNT/A. The specificity and sensitivity are comparable to conventional mouse lethality assay. The test has not been validated for microbiological examination and epidemiological investigations.

Another method alternative to the reference method is a rat test in which a direct measure of neuromuscular signal transmission, the compound muscle action potential (CMAP), was used to quantify BoNT concentration. The potential is generated by the contraction of muscle fiber and changes in the resulting micro current upon BoNT treatment. The micro current generated by muscles is correlated with BoNTs concentration. In the literature, the use of this method for BoNT/A, C and E detection was described. The limit of detection for mentioned toxotypes is lower than 1 MLD_{50}. These methods can estimate toxin quantity with significantly fewer animals required than the mouse lethality assay, however the main disadvantage of this technique is that toxin type must be known before examination (Torii et al. 2009).

The U.S. Food and Drug Administration recently approved a BOTOX® Cell-Based Potency Assay (CBPA) elaborated by Allergan Inc. for using as a replacement of mice bioassay (http://agn.client.shareholder.com/releasedetail.cfm?ReleaseID=587234). Cell-based assays measure BoNT receptor binding, translocation and enzymatic activity. A number of different neuronal and non-neuronal derived cell lines have been generated for use in BoNT assays. In the past: rat spinal cord cells, chick embryo neuronal cells, neuroblastoma cells N2A and BE(2) – M17 cells were elaborated (Eubanks et al. 2007).

Another *in vitro* test which could replace mouse bioassay is neuronal cell-based assay (NCB assays). These assays provide a model for BoNT detection which requires all steps of the cellular intoxication including cell surface binding, endocytosis, translocation of the light chain (LC) of botulinum toxin into cellular cytosol, and enzymatic activity of the LC on SNARE substrates. Recently described NCB assays exceed the sensitivity of mouse bioassay (Basavanna et al. 2013). Irrespective of the source of cells, all NCB assays require incubation of the cells with BoNTs for a defined time period, followed by the removal of the toxin and a quantitation of the endpoint for determining toxin activity. Among the most specific endpoints for BoNT activity is SNARE cleavage, and this can be used in any neuronal cell type. The using of NCB assays has some kind of limitation which, in comparison to *in vivo* tests, are: distribution, clearance, diffusion, transport, and other parameters. The most important application of NCB assays is determination of the potency of BoNTs, quantitative detection of neutralizing antibodies and use as research models. The NCB assays applications on foodstuffs or in field samples analysis require isolation and purification of BoNT (Pellet 2013).

Immunological methods

In comparison to the biological test, BoNTs detection with immunological methods is less labour- and time-consuming. Although, many of the earliest assays, such as radioimmunoassay (Boroff et al. 1973, Ashton et al. 1985), gel diffusion assay (Miller and Anderson 1971, Ferreira et al. 1981,), passive hemagglutination assay and the early applications of enzyme-linked immunosorbent assay (ELISA) (Notermans 1978, Dezfulian and Bartlett 1984) have poor sensitivity or specificity, the recent developments in signal amplification has enabled sensitivity equal to that of the mouse bioassay.

Enzyme-linked immunosorbent assay (ELISA) is the most frequently used immunological test for the BoNT *in vitro* detection. Typical sandwich ELISA setup is based on binding BoNT by specific antibodies. If BoNT is present in the sample, antibody conjugated to a suitable reporter binds specifically to the immobilized toxin. The reporter enzyme converts a chromogenic substrate into a colored product which is spectroscopically quantified and signal is compared to a standard calibration curve and the toxin quantity is calculated. Essential drawback of standard ELISA is sensitivity, because it is from 10 to 100 times lower than mouse lethality assay (Ekong 2000). The sensitivity of traditional sandwich ELISA could be upgrading by using biotin – labeled antibodies (Scother et al. 2013).

The ELISA modification based on lanthanide chelate labels with unique fluorescence properties is time-resolved fluorescence method (TRF) and gives much more reliable results. The long fluorescence decay time allows measurement of fluorescence after the background fluorescence has fully subsided (Peruski et al. 2002). After the immunoreactions, the lanthanide label is dissociated and rapidly forms a new, highly fluorescent and stable chelate inside a protective micelle with the components of the enhancement solution. Such characteristics contributes to the high sensitivity and low backgrounds characteristic of such assays. The limit of detection achieved by this method ranges from 4 to 20 pg/ml.

Another ELISA modification makes use of enzymatic activity of BoNTs and their substrates (SNAP-25, synaptobrevin) (Halis et al. 1996). Exploitation of BoNT endopeptidase activity guarantees high specificity of the assay and decreases the possibility of cross-contamination by other bacterial toxins. Endopeptidase-ELISA has been described for A, B, D, E, and F types, however commercial assay is available only for BoNT A detection (Hallis et al. 1996, Ekong et al. 1997, Schimdt et al. 2001). The endopeptidase-ELISA has a potential to replace the reference method because it detects only biologically active BoNT and detection limit of the assay ranges from 0,6 – 4,5 ng/ml.

Electrochemilumiscence immunoassay (ECL) is a technique similar to ELISA (Phillips and Abbott 2008). It differs from ELISA by reporter antibody, which has an electrochemiluminiscence tag and becomes luminescent in the presence of an electric potential. Depending on the matrix and BoNT type, the detection limit of the assay slightly varies, however it is generally lower than ELISA sensitivity (Guglielmo-Viret et al. 2005). Additional advantages include easiness in use and fast sample preparation. The using of electrochemiluminescence assays has some limitations. Most of presented results show a cross-reactivity among particular BoNTs. Recently, Sachdeva et al. (2013) described using electrochemiluminescence assay for screening of *Clostridium botulinum* outbreak strains associated with type A botulism. They obtained limit of detection of 40 pg/ml without cross-reactivity effect. Bok et al. (2013) demonstrated a new method with intrinsic fluorescence signal amplification for highly sensitive detection of BoNT/A. They obtained limit of detection at the level of 21.3 μg/ml.

In recent years, the ALISSA (assay with a large immunosorbent surface area) tests which are modification of ELISA have been described (Bagramyan et

al 2008). This assay is conducted in two steps. The first, an antibody-mediated step is a concentration of toxin onto a large bead surface. Subsequently, captured toxin molecules are subjected to a SNAPtide protease assay. The ALISSA enables detection of even 50 μg toxin/ml, significantly lower than with using the mouse bioassay or ELISA (Bagramyan et al. 2008).

In the literature, multiplex detection antibody system was described, which has been elaborated to analyze multiple epitopes on single targets in one sample. In this kind of methods, monoclonal and also polyclonal antibodies for reducing of non-specific results are used. Luminex xMAP® technology, for which microsphere beads conjugated with antibodies are used, is based on this kind of methods. This antibody – bead complexes enables detection of multiple epitopes in single sample, *e.g.* such technology was utilized for detection of botulinum toxins, ricin, abrin and staphylococcal enterotoxins in spiked food samples (Garber et al. 2010).

For BoNTs detection, the lateral flow tests which are a immunochromatographic assays relying on antibody-BoNT complex detection resulting in color change of the strip, like commercial pregnancy tests, are also popular. Lateral flow tests are inexpensive, easy to use, generate a visual read-out with no equipment needed and are very fast (15 min). Unfortunately, their sensitivity is lower in comparison to ELISA and other modern immunological methods. Limits of detection range from 5 to 50 ng/ml (Sharma et al. 2005, Chiao et al. 2008). This kind of tests could be used for rapid screening. However, recently described results demonstrate possible application of lateral flow tests with monoclonal antibodies. The specific detection of BoNT/A and B with using F1 – 51 and BoB – 92 – 32 antibodies has been presented by Ching et al. (2012) They obtained limit of detection of 10 ng/ml for mentioned toxotypes.

Chromatography- and mass spectrometry-based methods

Recently, chromatography- and mass spectrometry- based methods are also becoming much more frequently used assays, where peptides present in a sample are preliminarily separated by liquid chromatography and then they are detected by mass spectrometry (Klaubert et al. 2009). The finding that light chain of BoNT is a zinc-protease that cleaves proteins of SNARE complex at a neuromuscular junction, was essential for the development of *in vitro* assays based on BoNTs enzymatic activity. For detection of bacterial toxins, which are proteins, usually matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS) is used. The concentration of the protein in the sample is directly correlated with mass spectra and TOF analyzer measures time of flight of gas-phase ions from the ionization source to the detector, which is characteristic for each of the ions (Barr et al. 2004, Baar et al. 2005, Boyer et al. 2005). Imposing is also sensitivity of this technique which is capable of detecting 5 pg/ml of BoNT A, B, E and F (10-100-fold higher sensitivity than the mouse bioassay), relatively short time (16 hours) required for the examination and fact that only active BoNT is detected (Čapek and Dickerson 2010, Alam et al. 2012). Recently, Boyer et al. (2011) presented a method which could be used for a multiplex detection of toxin BoNT/A, B, C, D, E and F. The obtained level of detection was 20 times higher than mouse bioassay and was 0,05 LD_{50}. Identification of amino acid sequences specific for each protein occurring in nature creates new opportunities for BoNT detection by modern MS methods.

PCR based methods

Another type of methods which enable a direct detection of *C. botulinum* (without toxin detection) are based on molecular biology. Application of the mentioned methods makes *C. botulinum* isolation omitting possible. Polymerase chain reaction (PCR) and Southern blot hybridization are the methods most often described in the literature as regards to this species. Both techniques are characterized by high sensitivity and specificity. DNA of *C. botulinum* may be detected in sample directly (without enrichment process, *in situ*) or at different stages of culturing. There are known protocols based on nontoxic nonhemagglutinin gene (*ntnh*) detection coding nonhemagglutinin component of botulinum protoxins, common in all toxotypes of *C. botulinum* species (Raphael and Anreadis 2007). However, most of PCR protocols are based on *bont* genes detection, which determine active components of botulinum protoxin (BoNT) production specific for particular toxotypes (Aranda et al. 1997, Akbulut et al. 2005, Anon 2008). There are PCR procedures based on single *bont* gene detection (Sciacchitano and Hirshfield 1996, Yoon et al. 2005) and also on multiplex PCR detecting several toxotypes simultaneously (Szabo et al. 1994, Lindström et al. 2001, Akbulut et al. 2004). There are also described protocols based on Real-time PCR technique with using SybrGreen dyes and TaqMan molecular probes (Fach et al. 1995, Aranda et al. 1997, Braconier et al. 2001, Anniballi et al. 2012).

The sensitivity of PCR is highly influenced by the type of DNA matrix. The higher sensitivity is noticed when used DNA is obtained in the extraction and purification process, than after thermolysis of vegetative cells. Preparing DNA with extraction and purification is time-consuming, whilst thermolysis of cells suspension could be limited to 1 hour (Linström and Korkeala 2006).

A high sensitivity characterizes nested PCR test which is based on conducting reactions with using additional pair of primers. In the literature such kind of tests have been described for *C. botulinum* A, B, E and F toxotypes (Kakinuma et al. 1997, Fletcher et al. 2008). The usage of nested PCR enables to shorten time of enrichment in culturing process or detection of this pathogen in sample. The advantages of using this technique were described by Kakinuma *et al.* who gave an example of direct stool samples analyses from infant botulism cases with culturing process omitting (Kakinuma et al. 1997). Some ingredients of templates utilized in the detection process like fats, multimolecular proteins, immunoglobulins and natural microflora of samples might cause the inhibition of PCR (Al-Soud and Rådstöm 2001).

Generally, natural samples contamination level by *C. botulinum* is very low – it ranges from 10 to 1000 spores/kg, hence usually they are submitted to the enrichment process. The time of incubation is very important, when it is too short then it might be impossible to obtain sufficient amount of DNA for PCR analysis, which is linked with false negative results. Too long period might be result with cell lysis and degradation of DNA or appearing of thermoresistant spores. The optimal incubation time should range from 2 to 5 days (Saeed 2004, Lindström and Korkeala 2006).

The disadvantage of classic PCR is possibility of DNA detection originating from dead cells (Wolffs et al. 2005). This problem might be partly resolved by using enrichment process of sample. The other way for detection of viable cells might be RNA analysis by using RT-PCR (reverse transcription PCR). This reaction makes detection of gene expression possible, however obtaining RNA with proper quality is time-consuming. In literature such kind of tests was described for *C. botulinum* types A, B and E (McGrath et al. 2000, Lövenklev et al. 2004).

Nowadays, more frequently protocols based on Real-Time PCR with SybrGreen and molecular TaqMan probes are described. These methods are characterized by a higher sensitivity than classic tests based on PCR technique. Through possibility of melting temperature analysis and using, besides primers, fluorescently labeled molecular probes, it is capable to obtain high specificity and the shorter time of analysis. The available detection level by using Real – Time PCR is relatively low in comparison to the conventional PCR. This technique enables DNA detection in the amount corresponding to a few or tens *C. botulinum* cells (mass of *C. botulinum* genom equale app. 4 μg) (Raphael and Anreadis 2007, Grenda and Kwiatek 2010, Hill et al. 2010, Anniballi et al. 2012).

Development of molecular biology methods significantly improved *C. botulinum* detection and laboratory diagnostic of botulism. PCR methods enabled to shorten analysis time and the specific detection of the anaerobe DNA without isolation process. These methods are especially important for toxotype C and D, which are responsible for most animal botulism cases, and their toxicity is conditioned by infection by bacteriophage which contain toxicity genes of this anaerobe. Our laboratory practice confirmed that during passages of culture procedure, bacteriophage and its gene conditioning toxicity of *C. botulinum* was commonly lost, leading to false negative results. Usage of PCR-based methods enabled or strongly enhanced our efforts on botulism diagnosis in waterfowl (Grenda and Kwiatek 2009).

Immuno-PCR methods

In the literature also methods connecting PCR with immunological detection were described. In the immuno-PCR (I-PCR) the reporter antibody is a DNA-antibody conjugate with DNA used as an amplifiable tag. Amplification of DNA is performed either by normal PCR, which requires agarose gel electrophoresis detection of the PCR product, or more conveniently, by Real-Time quantitative PCR which is capable of direct DNA quantification using fluorescent dye labeling of the formed PCR product. Immuno-PCR technology greatly extends the sensitivity of immunoassays. This hybrid technology exhibited analyte detection from 100- to 1000-fold better than the ELISA method performed with the same antibodies. In general, immuno-PCR technology provides the basis for a new generation of sensitive immunoassays and may be useful in diagnosis of botulism and *C. botulinum* detection (Lindström and Korkeala 2006, Nakamura et al. 2013). The connection of immunological and PCR method could be used for detection of C and D strains and their toxins which have mosaic structure (BoNT/CD and DC). This would not be possible by using conventional PCR or immunological methods (Nakamura 2013).

Genotypic characterization of *Clostridium botulinum*

Clostridium botulinum genotyping enables epidemiological investigations in the botulism cases. Such methods are very important tool for evaluating the similarity between strains in the range of species and make their characteristic and distinguishing of genetically similar groups possible.

The most frequently used genotyping method considered as "gold standard" in molecular typing is macrorestriction analysis with using Pulse Field Gel Elecrophoresis (PFGE). This method is based on digestion of genomic DNA with specific restriction enzymes. After digestion characteristic DNA fragments are obtained. The number and size of them depend on the length of target sequences for used enzyme and on the percent of GC pairs. Rare-cutting endonucleases are used most frequently. The length of obtaining DNA fragments ranges from 0,5 kbp to 1000 kbp, which are subsequently separated electrophoretically in the variable electric field (pulse field). The separation is based on forced changing of DNA molecules directions. After electrophoretic separation, the patterns of entire genomic DNA are obtained which create image of bacterial chromosome structure. This method has a very high potential. The comparison of obtained patterns make similarity determination of strains submitted to the analysis possible (Dingwall et al. 1990, Lindstöm and Korkeala 2006). Despite high possibilities of strains differentiation this method is very labour-consuming and difficult to carry out. DNA susceptibility to the action of extracellular nucleases, which could disrupt genotyping with using of standard protocols, especially in the analysis of *C. botulinum* group II, is of a great importance. (Lin and Johnson 1995, Johnson et al. 2005, Nevas et al. 2005).

The other kind of method which enables genetic characterization of microorganisms is rybotyping. This technique is based on highly conservative genes analysis coding ribosomal RNA occurring in all bacterial organisms. Genes coding particular type of rRNA are separated thanks to polymorphic regions, which characterize high diversity of their sequences and length. These genes are a very good material for filogenetic analysis. Also, as in the case of PFGE, susceptibility of DNA to the action of extracellular nucleases, which cause some limitations in rybotyping use, is very important (Grimont and Grimont 1986, Skinner 2000). An automatic system RiboPrinter (Qualicon, Wilmington, DE), which has been also used for *C. botulinum* strains genotyping (Skinner 2000) is widely used in ribotyping analysis.

Another method which has been used for genotypic analysis of *C. botulinum* is amplified fragment length polymorphism (AFLP). This method is based on 2 stages. First, digestion of genomic DNA with two types of restriction enzymes is conducted. Subsequently, ligation of the obtained fragments with adequately designed adaptors is performed. The ligation products are submitted to selective amplification with using homologic primers to the adaptor-ligand sequence. In literature the usage of AFLP technique with using Hind III and HpyCH4IV enzymes has been described. This method constitutes an excellent tool for filogenetic analysis and enables differentation between I and II mebotabolic group of *C. botulinum* (Vos et al. 1995, Lindström and Korkeala 2006).

For the genotypic analysis of *C. botulinum* also Rep-PCR technique is used, which enables finding of repetitive sequences in genomes. The using of this technique to the genotypic analysis of *C. botulinum* is limited. In the literature the usage of Rep-PCR for the toxotypes B and E belonging to II metabolic group was described. The using of this method for the characterization of I metabolic group was only reported in the case of toxotype F (Versalovic et al. 1991, Hyytia et al. 1999).

Recently, DNA microarrays technology and MLVA method (Multi – locus variable number tandem repeat analysis) are adopted for *C. botulinum* characterization. Microarray test was performed by Raphael et al. (2010). The authors created microarray with 62 different sequences based on known variable regions in the genome of *C. botulinum* ATCC 3502. This array was applied for differentiation of *C. botulinum* toxotype A strains. The MLVA method is based on amplification of a variable number of tandem repeat regions using PCR. Generally, the tandem repeat regions are enumerated by the size of the PCR amplicon for each locus. MLVA has been used increasingly as a molecular typing method for various bacterial species as alternative to PFGE (Fillo et al. 2011, Umeda et al. 2013).

The consumers' demands of preservatives-free and low-processed food create challenges for searching new methods for *C. botulinum* elimination. A great attention focused on food and feed microbiological safety is the reason for researches for application of PCR methods for *C. botulinum* detection in above – mentioned matrixes by the European Committee for Standardization (Anon 2008). Nowadays in Poland, animal botulism and (sometimes) human botulism are generally diagnosed by mouse lethality assay (reference method according to AOAC). Supplementarily in our laboratories, PCR methods are used, which enable detection of DNA specific for *C. botulinum* species and its six toxotypes: A, B, C, D, E and F (Grenda and Kwiatek 2009). Taking into account disadvantages of above mentioned methods for

BoNT detection and animal or human safety in this aspects, also method based on enzymatic activity of BoNTs with use of MS is developed at the NVRI in Pulawy. According to the authors opinion, in the near future, the results of the mentioned research enable a proper control of *C. botulinum* and botulinum toxin occurrence in food and feed. This research will make also better laboratory diagnostic of botulism in humans and animals possible.

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