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Animal Botulism Outcomes in the AniBioThreat Project

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Botulism disease in both humans and animals is a worldwide concern. Botulinum neurotoxins produced by *Clostridium botulinum* and other Clostridium species are the most potent biological substances known and are responsible for flaccid paralysis leading to a high mortality rate. *Clostridium botulinum* and botulinum neurotoxins are considered potential weapons for bioterrorism and have been included in the Australia Group List of Biological Agents. In 2010 the European Commission (DG Justice, Freedom and Security) funded a 3-year project named AniBioThreat to improve the EU's capacity to counter animal bioterrorism threats. A detection portfolio with screening methods for botulism agents and incidents was needed to improve tracking and tracing of accidental and deliberate contamination of the feed and food chain with botulinum neurotoxins and other Clostridia. The complexity of this threat required acquiring new genetic information to better understand the diversity of these Clostridia and develop detection methods targeting both highly specific genetic markers of these Clostridia and the neurotoxins they are able to produce. Several European institutes participating in the AniBioThreat project collaborated on this program to achieve these objectives. Their scientific developments are discussed here.

Botulism is a severe flaccid paralysis disease caused by neurotoxins produced by the Gram-positive anaerobic spore-forming bacteria *Clostridium botulinum* and some strains of *Clostridium baratii* and *Clostridium butyricum*, which are distributed ubiquitously in soil and aquatic environments.[1] They are divided into 7 toxin types (A to G) depending on their antigenic properties.[2] Toxin types A, B, E, and F, produced by *C. botulinum* group I (proteolytic) and group II (nonproteolytic), are mainly responsible for human botulism. Toxin types C and D, produced by strains belonging to group III, are more associated with animal botulism.[3] Group IV, also referred to as *C. argentinensis*, has not generally been associated with illness.[4] *C. baratii* (Type F) and *C. butyricum* (Type E) can

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also produce botulinum neurotoxins F and E, respectively.[5,6] Some *C. botulinum* strains producing 2 different toxins or 1 toxin and carrying a silent toxin gene have also been reported.[7]

Biological threats have been given much attention since the Biological Weapons Convention.[8] Because of their extreme potency, the botulinum neurotoxins (BoNTs) are classified as high-risk threat agents for bioterrorism.[9] Clostridium BoNTs have been included in the Australia Group (AG) List of Biological Agents.[10] The Centers for Disease Control and Prevention (CDC) in the United States also considers botulinum neurotoxins as a category A agent that could pose a risk to national security.[11] The deliberate release of aerosolized botulinum neurotoxin or contamination of the feed or food chain for the purpose of bioterrorism is a concern.[9,12]

Botulinum neurotoxin–producing bacteria can cause serious problems in wild and domesticated animals such as birds, cattle, horses, sheep, and mink. Outbreaks with high mortality in waterfowl and poultry have become an increasing environmental and economic problem.[13] The risk of using animal botulinum neurotoxin–producing bacteria or toxins for agroterrorism is of concern to the European Union (EU). The availability of rapid and specific methods for testing BoNT-producing Clostridia is a prerequisite for establishing monitoring programs to track and trace back *C. botulinum* contamination in animals and foodstuffs. Faster detection of BoNT-producing bacteria can also result in a shortening of the outbreak by means of vaccination or removing the animals and sanitation and/or closing the farm to prevent further spread of the bacteria.

To improve monitoring and threat assessment based on threat recognition, the European project AniBioThreat has focused in particular on risks concerning animal bioterrorism. AniBioThreat laboratories from France (Anses), the Netherlands (CVI, RIVM), Denmark (DTU), Italy (ISS, IZSVe), and Sweden (SVA) have taken part in the fight against bioterrorism by improving tracking and tracing of accidental and deliberate contamination of *C. botulinum* in the feed and food chain. To achieve this objective, *Clostridium botulinum* group III has been characterized by sequencing, and spectrometric and nucleic acid–based detection methods have been developed. It should allow appropriate detection of and response to outbreaks associated with accidental and deliberate contamination of feed and food with BoNT-producing Clostridia. In addition, such methods could help in isolating *C. botulinum* strains for further characterization with the aim of providing epidemiologic information.

Strain Characterization of *C. botulinum* Group III

The National Veterinary Institute of Sweden (SVA) aimed to increase the knowledge about genetic diversity and genomic content of European *C. botulinum* physiological group III strains by using different fingerprinting and whole-genome sequencing methods. The purpose of collecting strain characterization information was to create a reference database that can be used for epidemiologic purposes—for example, during an investigation of a botulism outbreak. Strains belonging to *C. botulinum* group III are known to be difficult to isolate, and very little work has previously been performed regarding characterization of this group of strains.[13]

One objective was to isolate, characterize, and subtype *C. botulinum* group III strains by molecular fingerprinting techniques.[13] Avian samples were collected from different parts of Europe, and strains of *C. botulinum* type C/D were isolated. The strains were subtyped using 2 fingerprinting techniques that were first optimized for these strains: pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA analysis (RAPD). Subtyping revealed that the strains could be divided into 2 major subgroups, indicating that the genetic diversity of European avian *C. botulinum* group III strains is low.[13] Both techniques worked well after optimization and gave the same subtyping result. However, the results from PFGE were easier to interpret, whereas the results from RAPD were acquired faster.

The next objective was to analyze the genome of *C. botulinum* group III strains. DNA was subjected to whole-genome sequencing using Roche 454 technology. In this project, the first complete genome of a *C. botulinum* group III strain was published, and it revealed a genome with dual identity.[14] On one hand, it belongs to the pathogenic species *C. botulinum* since it has the ability to produce the botulinum neurotoxin, but on the other hand, as a genotypic species, it shows higher genomic similarity to *C. novyi* and *C. haemolyticum* than to the other groups of *C. botulinum*. It appears that genotypic species share a conserved chromosomal core but can be transformed into various pathogenic variants by modulation of the highly plastic plasmidome. Analysis of genomes of *C. botulinum* revealed that the majority of toxin genes, including the *bont* cluster, are plasmid-borne.[15] The genomes analyzed from group III contain an unusually high number of plasmids carrying different toxin genes. Some of these genes showed high similarity to genes coding for different toxins in other *Clostridium* species, and some toxin genes have moved between different plasmids within the same physiological group. This indicates that horizontal transfer of toxin genes is taking place within and between *Clostridium* species.

Upon comparing genomes between closely related strains from the same subgroup, it was revealed that the biggest differences between strains were not only the set of plasmids they contained, but also the number and location of mobile genetic elements within the genomes. Characterization of plasmid content and location of mobile elements in the genome can be used to identify and trace strains since this information can complement and narrow the result given by established fingerprinting techniques such as PFGE and RAPD.

Animal Botulism Detection Method Development

Since botulism is a life-threatening condition, a rapid diagnosis is essential for successful therapy. There are 2 options to perform the diagnosis: identify the toxin or the bacteria. The mouse lethality assay followed by sero-neutralization to identify the 7 existing toxin types has remained the standard method for detecting toxin in serum or feces.[16] However, the mouse bioassay presents several drawbacks, including requiring at least 4 days and the ethical issue of a large consumption of laboratory animals.[17] The cost of the mouse test—an average of 35€ (internal estimation) including laboratory office, staff, and consumables—could also be a major disadvantage. Thus, there has been a great need for a faster, equally sensitive method that is not based on animal use. Comparison of laboratory detection methods and sensitivity previously published by Lindström and Korkeala[2] showed that a different in vitro assay could replace the mouse test. In the AniBioThreat project, we aimed to develop and improve both toxin and bacteria detection methods. Their development results are described here.

Endopep Mass Spectrometry

The development of a comparable alternative analytical method to the mouse bioassay has not been a straightforward procedure, owing to the chemical nature of the botulinum neurotoxins in combination with their extreme toxicity. Very high detection sensitivity and specificity, and the ability to exclusively measure the active toxin, are thus requirements for a new method to replace the mouse bioassay. Botulinum toxins can be detected by a variety of techniques, including enzyme-linked immunosorbent assays (ELISAs)[18] or endopeptidase activity receptor-binding assay (EARB).[19] Another new concept is based on botulinum neurotoxin cleavage of synthetic peptides followed by detection of the product peptides by Matrix-Assisted Laser Desorption Ionization (MALDI) and/or Electrospray Ionization (ESI) Mass Spectrometry (MS).[20]

During the AniBioThreat project, the department of Chemistry, Environment, and Feed Hygiene at the National Veterinary Institute of Sweden has worked on implementation of the Endopep mass spectrometry (Endopep-MS) method for detection of BoNT/C, /D, and their mosaics form as an alternative test to the mouse bioassay. This method was developed by John Barr and his colleagues at CDC, who first published their work with BoNT/A-G in 2005.[20,21] Since then they have described extensive work improving the sensitivity for the BoNTs in biological samples such as serum and stool and reached limits of detection lower than those of the mouse bioassay for BoNT/B, /E, and /F and equal to the mouse bioassay for BoNT/A in serum.[22] A study to confirm botulism in birds and cattle by the Endopep-MS method has for the first time been applied to detect BoNT activity in selected clinical samples of animal origin, which previously tested positive with the mouse bioassay.[23] This method combines the biologic specificity of the BoNT enzymatic activity with the unparalleled detection specificity of mass spectrometry, which has for the first time been applied to detect BoNT activity in selected clinical samples of animal origin that previously tested positive with the mouse bioassay. This was the beginning of cross-validation studies between the 2 methods. Thus, Endopep-MS is anticipated to be an attractive alternative to the mouse bioassay.

Real-Time Polymerase Chain Reaction

Detection methods to identify BoNT-producing Clostridia by nucleic acid amplification methods are also rapid, specific, and reliable alternatives to biological techniques.[2,17] From collecting samples to the enrichment step, to nucleic acid extraction, to polymerase chain reaction (PCR) real-time assay, the detection process could be achieved in an estimated time of 24 to 48 hours, with an estimated average cost for such a molecular assay of roughly 1€ to 3€ per sample.

Considering the crucial role of DNA extraction in producing suitable PCR results, a comparison study was performed during the AniBioThreat project by ISS to evaluate 4 different DNA extraction methods. Eleven strains and 25 naturally contaminated samples were used in the comparison showing that Phenol-Chloroform-Isoamyl alcohol and the DNeasy Blood & Tissue kit produce the more intact DNA, while Chelex matrix and the DNeasy Blood & Tissue kit produce more amplifiable DNA, and NucliSENS® miniMAG produces the most pure genetic material but not a sufficiently intact yield.[24]

PCR-based tests offer a reliable alternative to screen bacterial colonies, pure liquid cultures, and sample enrichments for the presence of the neurotoxin encoding genes, indicating the presence of *C. botulinum* and other BoNT-producing Clostridia without the use of laboratory animals.[25,26] While they do not solve the shortcomings of the detection of bacteria instead of toxin, PCR-based assays have the advantage of being rapid, easy, and highly specific. A large number of studies have focused on the detection of *C. botulinum bont*/A, B, E, and F genes responsible for toxin production leading to human botulism.[7,25-35] There are also several reports on the detection of type C (*bont*/C) and type D (*bont*/D) genes by conventional PCR[36-42] and a few by real-time PCR.[43-47] Real-time PCR has the advantages of being highly specific and sensitive with no need of post-PCR processing, in contrast to conventional gel-based PCR. Through the AniBioThreat project, several institutes and laboratories investigated and developed new methods for detecting *C. botulinum* type C and D and their mosaic forms.

The National Swedish Veterinary Institute (SVA) has developed a real-time PCR assay for detection and identification of *C. botulinum* type C neurotoxin gene that also covers the chimeric C-D sequence as an alternative to the mouse bioassay.[48] The complete method consists of an optimized enrichment protocol followed by automated DNA extraction prior to real-time PCR. The sensitivity of the PCR assay was determined with purified DNA to be approximately 50 copies per PCR reaction. The specificity of the PCR assay was evaluated on a panel of about 30 relevant bacteria and on samples of caecum from birds collected in connection with botulism outbreaks on Swedish poultry farms.

With the aim of detecting and typing *C. botulinum* group III organisms, the Istituto Superiore di Sanità (ISS) developed and validated in-house a multiplex real-time PCR using SYBR Green.[49] Selectivity, limit of detection, relative accuracy, relative specificity, relative sensitivity, and repeatability of the method were investigated. The multiplex real-time PCR used showed a 100% selectivity, 100% relative accuracy, 100% relative specificity, 100% relative sensitivity, and a limit of detection of 277 and 580 DNA copies for *C. botulinum* type C and *C. botulinum* type D, respectively.

Based on the GeneDisc cycler platform (Pall GeneDisc Technologies), the French Agency for Food, Environmental, and Occupational Health Safety (Anses) developed 2 GeneDisc arrays for detecting and typing *C. botulinum* types C, D, and their mosaic forms.[45] The limit of detection of the PCR assays was 38 fg of total DNA, corresponding to 15 genome copies. Artificially contaminated samples of caecum showed a limit of detection below 50 spores/g. The tests were performed with a large variety of bacterial strains, including *C. botulinum* types C, C/D, D, and D/C; other BoNT-producing Clostridia strains; non-BoNT-producing Clostridia; and other bacterial species, and showed a high specificity. These PCR assays were compared to previously published real-time PCRs[43,44] for the detection of *C. botulinum* in 292 samples collected from cases of botulism events in 4 European regions. The correspondence of the results between this assay and reference real-time PCR[43,44] was 97.9%. Interestingly, only BoNT mosaics, types C/D and D/C, were found predominant in naturally contaminated samples, independent of their animal origin and geographical location.

Based on the molecular assays developed by Anses, the Central Veterinary Institute (CVI) in the Netherlands planned a survey study using the GeneDisc technology to perform a large epidemiologic investigation on mink feed samples and evaluate its use in the routine screening of mink feed. Following the results obtained with the GeneDisc arrays, a European ring trial was performed with 8 participating laboratories: Italy (ISS, IZS), France (Anses, LDA22), the Netherlands (CVI, RIVM), Denmark (DTU), and Sweden (SVA). The trial consisted of 33 strains and 48 clinical and food samples linked to botulism cases, which were analyzed using the 2 GeneDisc arrays previously developed.[45] Results showed concordance results between 99.35% and 100% for both GeneDisc arrays among the 8 laboratories.[50]

Conclusion

International organizations such as the World Health Organization (WHO), the Food and Agricultural Organization (FAO), and the World Organization for Animal Health (OIE) have in the past few years shown their concern about bioterrorism threats and have initiated several actions. Botulism is one of the most serious foodborne diseases and has been considered a public health emergency, and a deliberate contamination with neurotoxin and/or toxin-producing Clostridia remains a concern for the food safety authorities in Europe[51] and elsewhere. Standardized rapid, specific, and suitable methods to detect and type botulism toxins and BoNT-producing Clostridia form the basis for a prompt identification of the possible source and for the clinical management of the disease. Considering that different species of BoNT-producing Clostridia have been associated with botulism cases, detection of neurotoxins by mass spectrometry or identification of the toxin-producing bacteria by a real-time PCR approach to detect genes encoding BoNTs remain useful tools to counter bioterrorism threats. Through the European AniBioThreat project, we have successfully participated in developing tools to allow appropriate detection of and response to outbreaks associated with accidental and deliberate contamination of feed, food, and fecal samples.[23,45,48,49] These tests allowed the detection of animal normal or mosaic *C. botulinum* type strains and toxins, which sometimes are difficult to identify by the standard mouse bioassay. The methods developed in this project represent a significant improvement in the monitoring of *C. botulinum* contamination and prevention and can be used to reduce the number of animal tests, shorten diagnosis time, and decrease the cost of analysis. The next step to achieve rapid diagnostics of animal botulism is to implement those methods described here in laboratories around Europe. This work has been started in the AniBioThreat project, by providing ring trials. Further validation work is also required before the Endopep-MS method can be implemented in routine diagnostics and we finally can move away from the mouse bioassay.

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