Workshop on the risks associated with animal botulism and ANIBOTNET final meeting

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Abstract

A workshop on the risks associated with animal botulism was held in Maisons-Alfort, France in March 2019. The objectives were to debate the public and animal health risks related to botulism, to disseminate the results obtained from the ANIBOTNET project to other European research teams that were not involved in the project and to other stakeholders, and finally to strengthen and broaden the existing research network on this topic. The workshop was divided into 4 sessions: public and animal health risks associated with botulism, a specific focus on risks associated with agricultural biogas plants, dissemination of ANIBOTNET results, and network development and future perspectives. In all, 58 delegates from 13 countries attended the workshop. A summary of the main topics and discussions during the workshop is presented here.

Keywords

- Anaerobic digestion
- Animal health risks
- Biogas plants
- Botulism
- *Clostridium botulinum*
- Diagnosis
- ELISA
- Endopep-MS
- Epidemiology
- Foodborne disease
- Laboratory proficiency testing
- Next-generation sequencing
- One Health
- Public health risks

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Introduction

On March 28 and 29, 2019 a workshop on the risks associated with animal botulism was hosted by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) at its Headquarters in Maisons-Alfort, France. The workshop, funded by MedVetNet (2018_WS-1), included 8 presentations by invited speakers, 6 presentations by partners from the ANIBOTNET project, and 27 posters presented by delegates.

Background

Botulism is a neuroparalytic disease caused by botulinum neurotoxins (BoNTs), mainly produced by *Clostridium botulinum*. BoNTs affect both humans and animals worldwide. Botulism is included in list B of zoonoses and zoonotic agents in EU Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents (Authority *et al.*, 2018), i.e. the list of zoonoses and zoonotic agents to be monitored according to the epidemiological situation. Furthermore, BoNTs are included in category A of the US Centers for Disease Control and Prevention (CDC) list of potential biothreat agents (Centers for Disease Control and Prevention, 2012). The disease is rare but potential life-threatening in both humans and animals. Outbreaks in animals can be associated with high mortality and significant economic losses. On a worldwide basis, botulism is one of the most important diseases of migratory birds (Rocke, 2006). While surveillance data are available for humans, animal botulism is not listed among the 117 notifiable diseases monitored by the World Organization for Animal Health (OIE). In most European countries, animal botulism is not a notifiable disease, leading to a lack of animal botulism data regarding case numbers and outbreaks. However, a possible rise in the frequency of outbreaks was reported for France, Italy and some other European countries in the previous workshop focused on animal botulism that was held in Uppsala, Sweden in 2012 (Skarin *et al.*, 2013).

Objectives of the workshop

The purpose of this workshop was to discuss the public health risks associated with animal botulism. In addition, the workshop was used as an opportunity to share the results obtained from the ANIBOTNET project with other European research groups and stakeholders outside the project, and to strengthen and broaden the existing research network, fostering the One Health approach.

Animal botulism appears to be of increasing concern in some European countries (Skarin *et al.*, 2013), particularly in poultry flocks where it induces high mortality rates and economic losses, but also in other livestock productions including cattle, fur animals, and horses. Questions related to diagnosis, epidemiology, prevention, and control need to be explored to improve surveillance and management strategies. To address these questions, a 36-month (2016 – 2019) collaborative research project entitled ANIBOTNET was launched, with funding from the Animal Health and Welfare ERA-Net coordination actions.

The objectives of ANIBOTNET were to address knowledge gaps on animal botulism and to design strategies to improve surveillance, control, and prevention of the disease. The project aimed to improve an endopeptidase-mass spectrometry approach (Endopep-MS) to detect BoNTs in different types of matrices, standardize diagnostic tests, develop genotyping tools, develop knowledge about the epidemiology of the disease, and test vaccine and bio-control strategies.

The project involved 9 research groups from the EU (ANSES, IZSVe, IZSLT, ISS, FLI, RKI,

WUR, UH, and SVA) with complementary expertise in *C. botulinum*, BoNTs, mass spectrometry, veterinary diagnosis, genomic studies, epidemiology, and animal trials. It comprised five Work Packages (WPs). WP1 explored the stability, expression patterns, and regulation of BoNT type C, D, D/C and C/D genes in *C. botulinum* group III in vitro and in vivo. WP2 developed highly needed tools: Endopep-MS to detect BoNTs, Multiple Locus Variable Number of tandem repeats Analysis (MLVA) and MultiLocus Sequence Typing (MLST) for genotyping of *C. botulinum* isolates, and a workflow for mass spectrometric protein sequence analysis. WP3 aimed at better defining the epidemiological aspects of botulism. WP4 aimed at developing tools to consider strategies to prevent animal botulism. Finally, WP5 involved overall management.

The workshop delegates were risk management officers from local and national governments, veterinarians, scientists, laboratory technicians, PhD students, and company representatives from 13 countries: Belgium, Brazil, Denmark, Finland, France, Germany, Ireland, Italy, the Netherlands, Poland, Portugal, Sweden, , and the United Kingdom. An abstract book was drafted that included abstracts from the oral presentations as well as from posters that presented case reports or original research results related to botulism.

This report provides key information and summaries based on the presentations, discussions and abstracts provided in the abstract book.

Preamble of the workshop

ANSES Managing Director for Research and Reference Activities, Gilles Salvat, and Caroline Le Maréchal, Coordinator of the ANIBOTNET project and recipient of the MedVetNet workshop funding, opened the workshop. In addition to the presentation of the workshop programme, three recent outbreaks of botulism among wild birds in France in 2018 were presented and discussed. Importantly, they appear to have involved two different types of *C. botulinum* with type C/D and E neurotoxin genes detected by PCR. The detection of two different types of *C. botulinum* in three individual outbreaks among waterfowl raises questions for the first time about the emergence of additional risks to human health, as the identified type E is also pathogenic to humans.

Risks associated with botulism

The objective of this session was to discuss the human and animal health risks associated with botulism. This session started with a general overview of BoNTs and *C. botulinum*, covering recent original research related to mechanisms beyond BoNT production and *C. botulinum* sporulation, by Miia Lindström (University of Helsinki, Finland). To date, 7 BoNTs annotated as BoNT/A to G plus the recently described BoNT/X, and more than 40 subtypes have been described (Peck *et al.*, 2017). Six physiologically and genetically distinct clostridial species have been reported to be able to produce BoNTs: *C. botulinum* groups I, II, III and IV, *C. baratii* and *C. butyricum*. The botulinum neurotoxin is the most potent toxin known, with a human lethal dose potentially as low as 30–100 ng.

The session continued with a presentation by Cesare Montecucco (University of Padua, Italy) on the topic of non-clostridial species harboring the *bont* gene clusters. Some BoNT-like genes have been described in non-clostridial species such as *Weissella oryzae* (Mansfield *et al.*, 2015), *Enterococcus faecium* (Zhang *et al.*, 2018) or *Enterococcus* sp. (Brunt *et al.*, 2018), and *Chryseobacterium piperi* (Wentz *et al.*, 2017). The possible health risks related to these recently identified BoNT-like proteins were discussed during the presentation based on available knowledge: these toxins were identified with bioinformatic methods and were not associated with a disease or pathological status of any kind. It is not known whether these

genes are expressed or silent (Zhang *et al.*, 2017). Moreover, BoNT/En is not toxic in mice, suggesting mechanisms and targets different from those of the “classical” BoNTs.

The next presentation given by Mike Peck (QIB Extra Ltd, Norwich, UK) focused on human botulism, in particular foodborne botulism, which is the main form of the disease encountered in humans. Foodborne botulism is a severe intoxication caused by consumption of food containing BoNTs preformed by *C. botulinum*. It is a life-threatening disease with a mortality rate of 5–10 %, and has serious economic implications. Most foodborne botulism outbreaks are due to *C. botulinum* groups I and II producing BoNT types A, B, E and F. Group I and II strains are phenotypically very different, including growth requirements and heat resistance of spores, which suggests the need for informed risk assessment adapted to the characteristics of both groups, and the implementation of appropriate measures to prevent BoNT production by both groups. The application of safe production processes to prevent spore germination and further BoNT production is mandatory to ensure safe foods.

Luca Bano (Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Treviso, Italy) focused on animal botulism during his talk with a One Health perspective. Animal botulism is mostly due to BoNT types C, and D, and the mosaic forms C/D and D/C of *C. botulinum* group III. There are a few anecdotal reports on the involvement of these BoNTs in human botulism (Martrenchar *et al.*, 2019; Meyer *et al.*, 1953). Evaluation of the risk of contamination of farmed products during a botulism outbreak is a recurrent issue. Investigations conducted by Luca Bano on dairy farms during botulism outbreaks demonstrated that BoNT types C and D/C was not detected in tank or bovine udder milk. Botulism outbreaks are reported in wildlife, mostly wild birds and in livestock (poultry, cattle, minks, goats and sheep). In all, 40 outbreaks were confirmed by IZSVe between 2007 and 2018 in poultry, and 32 in cattle. A diagnostic strategy and epidemiological investigations were presented for both poultry and cattle production. Whole genome sequencing of strains involved in the outbreaks appears to be a reliable tool for epidemiological investigations.

The session ended with a presentation by Michel Popoff (Institut Pasteur, Paris, France) on the public health risks associated with botulism as a foodborne zoonosis. A review of available data on the possible transmission of botulism from animals to humans was presented, and the zoonotic potential of botulism, in particular during animal outbreaks with *C. botulinum* group III, was discussed. Only 11 cases of human botulism attributed to type C and one outbreak of botulism attributed to type D have been reported to date worldwide; however, unambiguous evidence is still lacking. BoNTs produced by *C. botulinum* group III have never been detected in human biological samples. Besides outbreaks, healthy carriage of *C. botulinum* by animals should also be taken into account. In most situations, human and animal botulism cases have distinct origins, and cross-transmission between animals and humans is a rare event. Considering the severity of this disease, human and animal botulism warrant careful surveillance. Effective identification and recording of animal outbreaks would help to better understand the epidemiology of botulism and putative links between animal and human cases. This is important when designing and targeting novel risk management measures*.

Briefly, this session showed that botulism is a severe disease more commonly encountered in animals than in humans. In the majority of cases, the BoNT serotypes involved in human and animal outbreaks are different. Foodborne botulism due to improperly processed or stored food remains the main form of botulism encountered in humans in Europe, while the origins of contamination in animals are variable: contaminated feed, litter, wrapped bales, poultry manure, carcasses, etc. As healthy animals can be carriers of different types of *C. botulinum* (BoNTs A, B, E, and F), there is a clear zoonotic potential which resulted in including botulism as a zoonotic disease in the EU framework (Directive 2003/99/EC). However, the zoonotic potential of botulism in animals induced by *C. botulinum* group III (BoNT/C, D, C/D, and D/C) has been debated. Considering the available data, it can only be concluded that cross-contamination between animals and humans has never been demonstrated.

* Rasetti-Escargueil, C., Lemichez, E., Popoff, M.R., 2020. Public health risk with botulism foodborne zoonoses. *Toxins*,12, 17.

C. botulinum, botulism, and agricultural biogas plants

The second session of the workshop was dedicated to the specific topic of the fate of *C. botulinum* during anaerobic digestion in agricultural biogas plants. Anaerobic digestion has become increasingly important over the last few decades. This process was initially applied for the treatment of sewage, but more recently has been widely developed for the treatment of agricultural by-products in Europe. The fate of pathogenic bacteria, in particular *C. botulinum* through anaerobic digestion, has been poorly studied up to now, and public concerns regarding the outcome of anaerobic pathogens during this process and their potential development have been reported.

Ute Messelhäußer (Bavarian Health and Food Safety Authority (LGL), Oberschleißheim, Germany) opened the session by presenting the background and challenges related to anaerobic digestion and botulism. Using germ carrier experiments, Ute Messelhäußer's group showed that BoNT-producing clostridia cells were reduced with D-values between 1.0 ± 0.2 d at 55 °C and 34.6 ± 11.2 d at 38 °C during anaerobic digestion. The experiments also showed on the basis of a two-year survey of eight Bavarian agricultural biogas plants that no BoNT-producing clostridia could be detected among the 154 investigated samples (Froschle *et al.*, 2015b). Based on these results, it was concluded that the risk of encountering BoNT-producing clostridia in biogas digestates is very low if good agricultural practices are applied, and that the pathogen is even reduced in the biogas process (Froschle *et al.*, 2015a).

Lorine Derongs (IRSTEA, Rennes, France) and Caroline Le Maréchal (ANSES, Ploufragan, France) presented the results of a one-year monitoring programme of three French agricultural biogas plants for the detection and enumeration of *C. botulinum* using an optimized protocol. *C. botulinum* was detected in 33 % of manure and 79 % of digestate samples. *C. botulinum* type B was detected in all positive samples. Enumeration of *C. botulinum* in both matrices yielded very low concentrations (below 10 MPN/g) in all samples. This study suggested that *C. botulinum* may be present at very low levels in some manures or digestates, but is very unlikely to present a risk of growth during anaerobic digestion.

Axel Mauroy (Belgian Food Safety Agency, Belgium) concluded the session by presenting the Opinion 26-2017 of the Scientific Committee of the Belgian Federal Agency for the Security of the Food Chain. The objective was to evaluate the animal health risks associated with spreading of manure or digestates contaminated with *C. botulinum* type D (D/C) on farmed land. A qualitative risk assessment was carried out, according to the methodology recommended by ANSES. The risk for animal health when spreading contaminated manure or digestate was considered by the SciCom to be 'very low'.

It can be concluded from this session that anaerobic digestion does not induce the growth of *C. botulinum* during the process, and that the contamination risk associated with spreading digestate is similar to that with spreading manure.

Dissemination of the ANIBOTNET project results

After a brief introduction presenting the objectives and background of the ANIBOTNET project, Fabrizio Anniballi (Istituto Superiore di Sanità, Rome, Italy) started this session with an overview of animal botulism in Europe. Although the number of reported outbreaks has increased, the epidemiological situation concerning animal botulism in Europe remains unclear. A systematic review and meta-analysis was performed to improve knowledge on the current situation, and on the burden of animal botulism in Europe. However, the eligible articles and related data were limited to a few countries. To overcome the gap in epidemiological knowledge, a survey was launched by Fabrizio Anniballi's group to collect data across European countries, so as to obtain an overview of animal botulism in Europe.

Yağmur Derman (University of Helsinki, Finland) presented the results obtained in WP1. The data suggested that various primary sugar carbon sources differentially supported the growth, sporulation, and BoNT production of Group III *C. botulinum*. Experimental evolution studies showed that the probability of Group III *C. botulinum* losing its BoNT gene-carrying phage and thus becoming non-toxic varies significantly by host strain. Reinfection was not detected, suggesting that the laboratory conditions used support non-toxic states of these bacteria. WP1 also included the development of an experimental model for avian botulism. A poster presenting preliminary results from a non-toxic model was developed in WP1.

Annica Tevell Åberg (National Veterinary Institute, Uppsala, Sweden), Martin Dorner (Robert Koch Institute, Berlin, Germany) and Fabrizio Anniballi (Istituto Superiore di Sanità, Rome, Italy) then presented the outcomes of WP2 ("Development of tools intended for botulinum neurotoxin detection and genotyping"). This WP aimed at delivering validated diagnostic methods that can replace the traditionally used mouse bioassay for the detection of BoNTs. Endopep-MS is one of the most promising methods for BoNT detection available, and a strong candidate to replace the mouse bioassay. One objective of WP2 was to modify and validate Endopep-MS for matrices other than serum samples. A protocol for BoNT detection in cattle, horse, chicken, and turkey liver samples was developed and validated within the project. The new protocol was successfully used to confirm botulism in several botulism outbreaks. Another strategy tested within WP2 was ELISA, which is an easy-to-use method that does not require sophisticated equipment like Endopep-MS. Strategically, ELISA could be used as a screening method that can be easily applied by routine laboratories, while Endopep-MS could serve both as a screening and confirmation method performed in expert laboratories. Within WP2, ELISA tests for the detection of BoNT C, C/D, D, and D/C were developed and validated on veterinary samples previously analysed by PCR.

Protein sequencing of BoNT was performed by mass spectrometry within WP2, and PCR-based genotyping tools, i.e. MLVA and MLST were developed.

An interlaboratory proficiency test was also organised within WP2, in order to evaluate the different methods used for BoNT detection. The full outcomes of WP2 were presented in a poster.

Fabrizio Anniballi presented the epidemiological aspects of animal botulism (WP3). Strains collected during the surveillance activities conducted by the project partners were submitted to genetic comparison using the molecular tools developed in WP2 (MLVA and MLST), with the aim of evaluating their genomic variability. MLVA, MLST and whole-genome sequencing (WGS) were also used to identify the source of contamination in some outbreaks. With the aim of collecting epidemiological data on outbreaks occurring during the timeframe of the project, a specific database was built.

A talk presenting WP4 (Evaluation of measures to prevent and control animal botulism) was given by Luca Bano. Three different aspects were addressed in this WP: evaluation of the antibiotic resistance of strains, evaluation of a recombinant vaccine to elicit a protective response in different animal models, and evaluation of the potential of lactic acid bacteria as an antagonist of *C. botulinum* or BoNT production. The susceptibility of 71 *C. botulinum* group III field strains collected within five European countries to 13 antimicrobial drugs was tested during the project, with two main purposes: (i) first purpose of the test was prudent use of antimicrobials in poultry; and (ii) second purpose of the test was to explore the possibility of setting up a selective medium for the isolation of *C. botulinum* group III. The efficacy of a recombinant vaccine was tested in cattle, and the immune response was compared to the efficacy of a commercial toxoid-based vaccine. Finally, the efficacy of 40 lactic acid bacterial strains was tested against 37 *C. botulinum* strains. High sensitivity of *C. botulinum* to lactic acid bacteria was observed. All tested strategies showed promising results and opened up interesting possibilities for field applications so as to prevent and manage animal botulism outbreaks.

This session was closed by a discussion of WP5. This WP aimed at drafting guidelines intended for the management of animal botulism outbreaks: clinical suspicion, sample collection, laboratory confirmation, outbreak classification, data collection, therapeutic measures, and sanitation procedures. Each section was detailed during the presentation.

Future perspectives and networking

The workshop was closed by discussions about future projects, research topics, and networking. As illustrated by the proficiency test results from WP2, methods for BoNT detection, *C. botulinum* typing and sequencing, *C. botulinum* group III isolation, and botulism surveillance need to be better disseminated and adopted, and corresponding training provided.

This workshop was the second organised on the topic of animal botulism and allowed the number of research groups attending the workshop to be extended. Maintaining this network of laboratories in Europe or in other countries and including stakeholders appears to be very important to keep this research programme dynamic, and to make further progress on the management of animal botulism.

Overall, the workshop presentations and discussions gave in-depth insight into new available tools and progress on this topic. Replacement of the mouse bioassay, which is still the gold standard for BoNT detection, is a high priority. The ELISA and Endopep-MS methods were in-house validated for new sample materials, such as organs, during the ANIBOTNET project in the context of animal botulism outbreaks in real conditions. The proficiency test organised was a great opportunity to overcome the lack of quality control measures available in the field, and to test existing and novel diagnostic approaches on blinded samples. While many laboratories returned satisfactory results, the proficiency test also identified gaps and training needs that need to be addressed in the future. Important in vitro methods like Endopep-MS (Bjornstad *et al.*, 2014) or ELISA (Hansbauer *et al.*, 2016), if proper training is provided, can offer the same or better performance compared to the mouse bioassay.

The ANIBOTNET project and the workshop also highlighted particular topics considered to be priorities by the workshop delegates, and these should be addressed in the future:

- Continue research work initiated as part of the ANIBOTNET project, in particular validation of the alternative methods to the mouse bioassay in new matrices such as milk, and exploration of the mechanisms promoting group III *C. botulinum* growth and BoNT gene expression, with a view to better preventing outbreaks
- Share tools, diagnostic strategies, and guidelines for outbreak management, in particular with veterinarians
- Increase awareness on existing and emerging threats associated with animal botulism in order to obtain more reliable data on animal botulism
- Implement systematic characterization of *C. botulinum* isolates involved in animal botulism outbreaks, using WGS for example, to reinforce surveillance at the European level
- Collect data in order to identify and prevent risks to public health, the environment and the economy, for instance by collecting data regarding the contamination of food of animal origin produced during a botulism outbreak, such as milk, eggs, and meat, or by extending the studies on biogas plants to other European countries.

Future funding will help to maintain this unique network and to address the needs identified.

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List of invited speakers

University of Helsinki	Miia Lindstrom
Quadram Institute	Mike Peck
Institut Pasteur of Paris	Michel Popoff
Istituto Zooprofilattico Sperimentale delle Venezie	Luca Bano
University of Padua	Cesare Montecucco
Bavarian Health and Food Safety Authority	Ute Messelhäußer
French National Research Institute of Science and Technology for Environment and Agriculture	Lorine Derongs
French Agency for Food, Environmental and Occupational Health & Safety	Caroline Le Maréchal
Belgian Food Safety Agency	Axel Mauroy

List of people involved in the ANIBOTNET project

ANSES: Caroline Le Maréchal, Marianne Chemaly, Rozenn Souillard, Sandra Rouxel, Typhaine Poezevara, Sabrina Macé, Laure Martin, Emmanuelle Houard, Patrick Fach, Cédric Woudstra

SVA: Kristian Björnstad, Mikael Hedeland, Ida Karlsson, Sofia Nyberg, Hanna Skarin, Annica Tevell Åberg

UH: Miia Lindström, Yagmur Derman, Cédric Woudstra

RKI: Martin B. Dorner, Martin Skiba, Ewa Schlereth, Heidrun Ranisch, Brigitte G. Dorner

WUR: Miriam Koene, Marc Engelsma, Yvonne Dijkstra

IZSVe: Ilenia Drigo, Tiziana Ferro, Angela Guolo, Elena Tonon, Luca Bano

FLI: Christian Seyboldt

IZSLT-ISS: Fabrizio Anniballi, Stefano Bilei, Concetta Scalfaro, Bruna Auricchio and Paola De Santis

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