Procedure and guidelines for the management of animal botulism

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Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation

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Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation

4 Introduction

Animal botulism is caused by botulinum neurotoxins (BoNTs) which block neurotransmitter release leading to life-threatening paralysis. The neurotoxins are produced mainly by the diverse group of *Clostridium botulinum* (Groups I-IV) but also by some strains of *C. baratii* and *C. butyricum*. So far seven historical serotypes (A–G) comprising more than 40 subtypes, are reported to exist. Animal botulism is most frequently caused by *C. botulinum* Group III strains producing BoNT/C and D and the mosaic forms C/D or D/C; nevertheless, other rarer serotypes can also cause disease. Botulism, in particular among livestock such as cattle and poultry, can cause substantial economic loss.

The ANIBOTNET project was funded by the 2015 ANIHWA call and conducted between 2016 and 2019. This project involved 9 European Union (EU) groups involved in botulism research (ANSES, IZSVe, IZSLT, ISS, FLI, RKI, WUR, UH, SVA) with complementary expertise in *C. botulinum*.

The objectives of the project were to address knowledge gaps in *C. botulinum* Group III and animal botulism to design strategies to improve the surveillance, control, and prevention of the disease. We aimed at improving the Endopep-MS approach to detect botulinum neurotoxins in different matrices, standardizing diagnostic tests, developing genotyping tools, improving knowledge on the epidemiology of the disease, testing a vaccine, and implementing a bio-control strategy.

The project comprised 5 Work Packages:

WP1 explored the stability, expression patterns, and regulation of types C, D, and C/D genes in *C. botulinum* group III in vitro.

WP2 covered development of highly needed tools: Endopep-MS to detect BoNTs, MLVA and MLST for genotyping *C. botulinum* strains, and designing the workflow for mass spectrometric protein sequence analysis.

WP3 aimed at better defining epidemiological aspects of animal botulism.

WP4 aimed at developing tools to improve strategies for preventing animal botulism.

WP5 involved overall management

One of the outcomes of the project was the preparation of guidelines for the management and handling of animal botulism outbreaks. The guideline objectives are to supply/provide veterinarians, stakeholders and scientific groups updated information regarding animal botulism useful for outbreak management. In these guidelines, ANIBOTNET partners tried to cover all steps of an outbreak, from clinical suspicion to cleaning and disinfection operations and disposing contaminated manure. The authors remain at the reader's disposal for more detailed information if required.

5 Generalities on animal botulism

Botulism is a neuroparalytic disease in humans and animals due to the action of botulinum neurotoxins (BoNT) that inhibit the transmission of nerve signals by preventing the release of acetylcholine. There are seven historically described BoNT serotypes annotated from A to G with more than 40 subtypes described (Peck et al., 2017). BoNTs are produced by *C. botulinum* but other *Clostridium* species such as *C. butyricum*, *C. baratii* and recently *C. sporogenes* have been recognized capable of producing BoNTs. In addition to these seven well-recognized toxins, the new types H (also referred as AH or FA) (Barash and Arnon, 2014) and X (Zhang et al., 2017) have been detected in strains originally classified as *C. botulinum* type B. Recently, BoNT-like genes have been found in non-*Clostridium* species, including *Weissella oryz*ae and various enterococci; however, the role and function of most of these BoNT-like genes remains elusive (Popoff, 2018, Azarnia Tehran and Pirazzini, 2018). Strains of *C. botulinum* are divided into 4 groups according to their physiology and BoNT type produced. *C. botulinum* group I and II that produce BoNT/A, B, E and F are mostly associated with human botulism. *C. botulinum* group III is part of the *C. novyi sensu lato* pathotype, producing BoNT/C, D, C/D and D/C, and is associated with animal botulism. *C. botulinum* group IV, which is able to produce BoNT/G, and is known also as *C. argentinense*, is suspected of being involved in a case of wound botulism (Taylor et al., 2010).

Botulism in humans is differentiated into three major forms: i) foodborne botulism, which is an intoxication caused by the consumption of food containing a preformed toxin, ii) wound botulism which is caused by an infection of wounds with *C. botulinum* followed by *in situ* bacterial growth with toxin production, and iii) infant botulism which occurs in babies less than 12 months of age and whose digestive microflora is not mature enough to be able to prevent *C. botulinum* from colonizing the digestive tract and producing toxins *in situ*. Apart from these three major forms, there are also two minor forms, namely, iv) iatrogenic botulism, as a result of medical or cosmetic procedures, and v) inhalational botulism, contracted by accidental BoNT inhalation via aerosols.

In animals, the forms of botulism are less well understood and the mechanisms behind the disease still need further study (initiation of an outbreak, pathogenesis, epidemiology, toxinogenesis of *C. botulinum* Group III, etc.). The clinical signs are caused by the action of BoNTs; however, whether the disease is due to the ingestion of preformed-BoNTs (intoxication), or to *in situ* BoNT production (toxico-infection) or a combination of both, has not been elucidated yet. During bovine outbreaks, when the largest number of cases in animals are encountered in the first week, the progression of the disease supports the uptake of a preformed BoNT toxin, much as found in foodborne botulism in humans. In poultry, it has been demonstrated that *in situ* production of BoNTs in the caeca, precedes the appearance of the disease (Miyazaki and Sakaguchi, 1978, Kurazono et al., 1988). It has also been demonstrated that ingestion of BoNT and *C. botulinum* through coprophagy is needed to develop botulism (Hyun and Sakaguchi, 1989). In poultry, another pathway is via the ingestion of preformed BoNTs through the cannibalization of carcasses or the ingestion of maggots, both serving as an additional BoNT source (Blandford and Roberts, 1970, Foreyt and Abinanti, 1980). The relative importance of these two last-mentioned pathways, which may coexist, is not known (Skarin et al., 2015, Haagsma and ter Laak, 1977). Epidemiological investigations have shown that *C. botulinum* can

be found in animal organs, in animal environments, in feed (Souillard et al., 2017, Souillard et al., 2014), and that identical isolates have been discovered from these different matrices (Myllykoski et al., 2009, Nakamura et al., 2010, Skarin et al., 2015). *C. botulinum* is commonly found in, and isolated from, the livers of broilers or cows showing clinical signs (Le Marechal et al., 2016a, Myllykoski et al., 2009, Skarin et al., 2010); however, the source of internal organ contamination and its role in the pathogenesis of botulism, is unknown. Nakamura et al. (2010) concluded that "these findings suggest that bovine botulism results from the ingestion of *C. botulinum* spores that germinate, multiply, and produce BoNT in the bovine gastrointestinal tract as well as infant botulism in humans" (Nakamura et al., 2010). However, the validity of this hypothesis has not been confirmed and remains a possible scenario; further studies are needed to better understand the pathogenesis of bovine botulism.

As for humans, wound botulism has been reported to occur also in animals (Liguori et al., 2008, Trampel et al., 2005). It has been assumed that botulism in foals is due to toxico-infection (Wilkins and Palmer, 2003) similar to what is observed in infants. But again, this is hypothetical and needs further study.

6 Clinical suspicion

C Diagnosis is based mainly on clinical signs, that is to say neuromuscular disorders and exclusion of other neurologic forms of disease (Anniballi et al., 2013b). Pictures or reports depicting typical clinical signs are available in some papers and are listed in Appendix 1.

Progressive symmetrical flaccid paralysis with recumbency is a typically diagnostic sign of animal botulism. Clinical symptoms can appear from 24 hours after exposure and manifest for up to 17 days (Anniballi et al., 2013b). The period between first appearance and persistence of clinical signs can be even longer, especially in cattle (Le Maréchal et al., 2019).

6.1 Clinical signs in birds

Botulism has already been reported in 264 bird species representing 39 families (Rocke, 2006). Anatidae are one of the family most commonly involved in outbreaks of botulism (Ventujol et al., 2017). In poultry production, the most common affected species and/or animal categories are broilers, turkeys, pheasants and to a lesser extent ducks, , laying hens, geese, and the guinea fowl (Le Marechal et al., 2016a, Le Marechal et al., 2017, Souillard et al., 2014, Ventujol et al., 2017, Bano, 2019).

Males seem to be more affected by botulism than females, especially in turkeys (Popp et al., 2012, Smart et al., 1983, Souillard et al., 2014).

Botulism is characterized by an ascendant flaccid paralysis that progresses from legs to the eyelids, and often is associated with respiratory distress (dyspnoea). The first clinical signs to appear in chickens affected by botulism is weakness of the legs (Bano, 2019). It would appear that the wing muscles are affected also during early onset of the disease. Sternal recumbency with closed eyes, reluctance to move, flaccid neck paralysis with inability to lift the head are typical symptoms (Sharpe et al., 2011). Birds are often unable to raise their heads, to walk or stand, to escape and are prostrate (Skarin et al., 2015). Birds are reluctant to move and they use the wings to balance the body during ambulation (Bano, 2019). Soiled vents have been reported in broilers and laying hens (Skarin et al., 2015, Dohms et al., 1982, Sharpe et al., 2011). In turkeys, the signs are less evident and usually the disease is suspected following a sudden increase in the mortality rate (Bano, 2019). Drowning of wild waterfowls is often seen to occur, due to neck paralysis and attendant inability to lift the head.

High mortalities are reported. For example, during investigations conducted in France, the average mortality was 13.9% (standard deviation: 9.4%) and the median was 10.6% (Souillard et al., 2014). Mortality rates typically range from 1% to 25% in poultry flocks (Dohms et al., 1982) and from 2.8% to 35.2% (Souillard et al., 2014) but higher mortalities are reported, up to 30% (Smart et al., 1983) or 50% in turkeys (Popp et al., 2012) and 84% in pheasants (Blandford, 1976).

Large outbreaks inducing high mortality levels (above 100 000 birds) have been reported in wild birds (Rocke and Bollinger, 2007).

6.2 Clinical signs in cattle

Botulism is more commonly reported in dairy cattle, but outbreaks in beef cattle occur also. Flaccid paralysis proceeds from the tail and hind legs to the head (Bano, 2019, Kummel et al., 2012) and vice versa (Braun et al., 2005).

At onset, there is a sudden drop in milk production; moreover, the faeces consistency of affected animals varies from dry and hard to diarrhoea (Kummel et al., 2012). Affected animals lie in sternal recumbency with the head on the ground or directed backwards into the flank, in a self-auscultation posture. In the initial phase of the disease, the abdominal muscles are flaccid, while the tongue is usually normotonic (Bano, 2019). Anorexia, ataxia, apathy, weakness, dysphagia, increased salivation, difficulty swallowing, paralysis, recumbency with head tucked into the flank, and dropping feed from the mouth, are the most common signs associated with botulism in cattle (Myllykoski et al., 2009, Kummel et al., 2012, Relun et al., 2017, Galey et al., 2000, Le Maréchal et al., 2019). Other symptoms are also frequently noticed: decreased ruminal contractions, reduced intestinal motility, tail paralysis, flaccid paralysis of tongue, paralysis of jaw muscles, respiratory difficulties, dry and hard faeces, or diarrhoea (Myllykoski et al., 2009, Kummel et al., 2012, Galey et al., 2000, Braun et al., 2005, Joubert et al., 1969, Prevot et al., 1955). Abdominal breathing in severely affected animals has also been described (Galey et al., 2000, Fjolstad and Klund, 1969). Hyperthermia is absent.
Usually, catastrophic losses are associated with bovine botulism, the mortality rate is often biphasic in its distribution (Bano, 2019): acute clinical signs are observed 72–96 h following exposure, other animals may show clinical signs 14-20 days after the onset of the disease (Neill et al., 1989, Myllykoski et al., 2009, Galey et al., 2000, Relun et al., 2017, Bano, 2019, Le Maréchal et al., 2019).

6.3 Clinical signs in horses

Clinical signs in horses are similar to those seen in cattle: the tongue may hang from the mouth, eyelid ptosis, anal and sphincter hypotonia, generalized muscle weakness, dysphagia, muscle tremors, a shortened gait, mydriasis with slowed pupillary reflex, in general all followed by recumbency, respiratory failure, and inability to rise (Johnson et al., 2016, Anniballi et al., 2013b).

Johnson et al. (2016) suggest performing the tongue stress test and the grain test, in which abnormal performance is indicative for botulism. This tongue test involves gently withdrawing the horse's tongue from the mouth while holding the jaws closed and assessing the horse's ability to retract the tongue (the expected result is retraction after 1 or 2 attempts). The grain test involves offering the horse 8 ounces of sweet feed in a flat feeding trough and timing the speed at which the horse consumes it (normal is below 2 minutes) (Johnson et al., 2016).

6.4 Clinical signs in fur animals

Outbreaks have been reported in the mink, the fox, and the ferret. The clinical signs are similar to those observed in horses and cattle: paralysis of the hindquarters, paraplegia, recumbency, wobbling, increased salivation, and laboured breathing (Lindström et al., 2004). A sitting position is observed in foxes with milder symptoms (Lindström et al., 2004). Death can occur extremely quickly in furred animals, less than 2-3 hours after the onset of clinical signs (Quortrup and Gorham, 1949).

6.5 Clinical signs in other animals

Some animal species are reported to be resistant to botulism like vultures and other carrion eaters (Ohishi et al., 1979), pigs, and cats. Outbreaks of botulism in pigs have been reported from Brazil on farms where the sanitary conditions are poor, with the pigs fed on the food waste obtained from restaurant and hotel kitchens (Raymundo et al., 2012). The reported clinical signs are anorexia, weakness, lack of coordination, locomotion difficulties, and onset of lateral decubitus. Involuntary urination and defecation, a reduction in the consumption of water and food was also observed (Raymundo et al., 2012). There is a single report involving botulism in cats fed pelican carrion positive for *C. botulinum* type C (Elad et al., 2004).

Fishes, sheep, goats, donkeys, turtles and dogs are reported to be sensitive to botulism, but few case reports are available.

7 Necropsy

There are no significant changes observed in the necropsy that can be considered pathognomonic of animal botulism. Animals affected by botulism show no gross or histologic lesions.

Secondary pathologies can appear in more protracted cases (Anniballi et al., 2013b).

8 Laboratory confirmation of botulism

Although diagnosis of botulism is mainly a clinical diagnosis and the signs are evocative for botulism, they cannot be considered specific, especially at onset; therefore, laboratory confirmation is required for a definitive diagnosis and is obtained by demonstrating one or more of the following (Anniballi et al., 2013b):

- The presence of BoNT in sera, organs, wounds, and/or faeces;
- The presence of BoNT-producing clostridia in organs, wounds, and faeces;
- The presence of BoNT or BoNT-producing clostridia in feedstuffs and/or poultry litter.

These diagnostic methodologies listed, can be used singly or in combination to confirm a clinical suspect of botulism. Each methodology, when applied singly, has limitations; these limitations can be overcome by using two or more methods in combination. However, depending on the sensitivity and specificity of the particular method used, the results may prove to be inconclusive. Each of the diagnostic laboratory methods applied are to a certain degree all susceptible to providing false negative or false positive results.
In the case of the use of the mouse bioassay, other toxic compounds, drugs, autoantibodies could be present in the clinical sample and be fatal to the mouse itself leading to a false positive result. However, performing serum neutralisation tests minimises the chance of false positive results in the bioassay. It not only identifies the BoNT type but can also help to distinguish between the presence of BoNT and heat-labile toxic compounds other than BoNT (in the latter case the antisera will not be protective).
This interpretive ambiguity applies also when using ELISA and again requires verification using other more powerful methods (see 7.1.2). The presence of proteases (e.g. trypsin) in the clinical sample matrix may induce false positive results when endopeptidase assays are used to detect BoNTs (see 7.3) because of non-specific degradation of the peptides, in particular in complex matrices. At present, only MS/MS methods can unambiguously show the presence of the toxin by supplying information about the protein sequence; unfortunately, MS/MS methods require sophisticated instrumentation and suffer from a mediocre sensitivity.

Detection of a *C. botulinum* organism commonly encountered in animal botulism (e.g. *C. botulinum* GIII) by PCR strongly supports initial clinical suspicion of botulism. However, it must be kept in mind that healthy animals (in particular cattle) could bring PCR positive enrichment cultures. For example, in a large study (1388 faecal samples), around 8% of individual faecal samples were positive across all farms or animal groups (either healthy or symptomatic animals (suspected visceral botulism)) when considering at the same time results obtained using 3 different protocols. Among the 109 positive

samples, 79.0% (86/109) were type A were 57.69%, 7.3% (8/109) were type B 11.54%, 8.2% (9/109) were type D 15.38, 0.9% (1/109) was type E 3.85% and 4.6% (5/109) were type F, 11.54% with no significant difference in the detection of BoNT genes between healthy and symptomatic animals (Fohler et al., 2016). It is noteworthy that BoNT was not detected in any of the faecal samples analysed in this case-control study using the mouse bioassay (Seyboldt et al., 2015) . It is also noteworthy that using the MCM procedure which is recommended for diagnosis (see 8.2), only 1.87 % faecal samples were positive (Fohler et al., 2016) and it should be kept in mind that faecal samples are one of the most relevant samples for the diagnosis of bovine botulism when using this procedure (Bano, 2019).Carriage of non-proteolytic type B *C. botulinum* (73%) and E and F (<5%) has also been reported in another study in cattle (Dahlenborg et al. 2003). Although a PCR is not a definitive proof it is the most reasonable approach to come to a timely diagnosis and in the situation of a very clear clinical picture the PCR detection is sufficient to diagnose botulism with high probability. In addition, PCR is the quickest method to assess the serotype involved in the outbreak and this remark is very important to exclude BoNTs serotypes usually responsible of human botulism. The low prevalence of PCR-positive samples collected from asymptomatic animals (Fohler et al., 2016; Dahlenborg et al. 2003) compared with the high prevalence detected in subjects with paralytic symptoms (Bano et al., 2017), make the PCR a precious diagnostic tool for botulism in cattle. Clinical picture, epidemiological investigations and PCR results should be eventually considered altogether to draw the final conclusion.

To overcome the multiple restrictions of the above-mentioned approaches, a combination of the different methods is advised to confirm the diagnosis of botulism (Dorner et al., 2013).

Depending on the animal species involved, some methods are weakly sensitive; for example, serum samples are often negative for BoNTs in cattle (Bano, 2019), turkeys (Le Marechal et al., 2016a) and horses (Johnson et al., 2014b). The use of PCR-based methods, or a combination of methods that detect both the toxin and the BoNT-producing clostridia, is recommended in particular for these animal species.

8.1 Methods available for BoNT detection

8.1.1 The Mouse Bioassay (MBA)

The current gold standard for BoNT detection in most countries is the Mouse Bioassay (MBA)(Stern et al., 2018). The method is based on intraperitoneal injection of a sample into mice. The diagnosis involves the observation of symptoms characterizing botulism in injected mice (fuzzy hair, muscle weakness, and respiratory failure, wasp-like narrowed waist). Due to the presence of other compounds that could induce death in mice, a MBA positive result must be confirmed using a serotype-specific antitoxin neutralization test that will also allow determination of the BoNT serotype involved. While this method is still widely used, it must be kept in mind that the method is ethically challenged, laborious and requires trained personnel (Lindström and Korkeala, 2006).

8.1.2 ELISA tests

In a sandwich ELISA the analyte (e.g. BoNT) is captured from a sample by the surface-bound antibody (capture antibody) and the bound BoNT is detected by another antibody, usually biotinylated (detection antibody). Streptavidin-enzyme conjugates are used to couple the detection antibody to an

enzyme such as peroxidase or alkaline phosphatase. The amount of enzyme is then measured in a colour, fluorescence or luminescence reaction. Analyte quantification is possible based on a standard curve of known analyte concentrations. The detection of BoNTs by ELISA has been used for more than 50 years. However, many approaches, in particular those based on polyclonal antibodies, lack either specificity or sensitivity or both. Polyclonal antibodies are a mixture of different antibodies recognizing the cognate antigen with various affinities and binding to different epitopes in addition to numerous other host antibodies directed against other unrelated antigens originating from previous encounters with other antigens. Due to this heterogenic nature, polyclonal antibodies can often lead to false positive results. Monoclonal antibodies can overcome this obstacle as they are directed only against one epitope of a given antigen and thus rarely cross-react against unrelated antigens. On the other hand, monoclonal antibodies are more sensitive towards sequence variations in their cognate antigen which could eventually prevent antigen recognition and lead to false negative results. Moreover, the chemistry used for signal generation in an ELISA often relies on the enzyme coupled to the detection antibody (e.g. peroxidase, alkaline phosphatase). Sample matrix components like peroxidase or peroxidase inhibitors can then interfere with the enzyme reaction. Additionally, samples with a high content of biotin or biotin-like molecules could artificially capture the streptavidin-enzyme conjugate and induce false positive results. Therefore, it is necessary to control ELISA for false positive e.g. by using an unrelated capture antibody in parallel, and for false negative results by spiking samples with a known concentration of analyte prior of detection.

To detect the *C. botulinum* Group III BoNT/C and D toxins, or their mosaic CD and DC forms, various ELISA methods including on-site tests have been developed that target individual Group III BoNTs (Brooks et al., 2011, Gessler et al., 2006, Klewitz et al., 2006, Nakamura et al., 2013, Notermans et al., 1982, Thomas, 1991); one research group has described an ELISA for detecting all four Group III BoNTs (Hansbauer et al., 2016).

8.1.3 Endopep-MS

In the Endopep-MS method, both the antigenic properties and specific proteolytic activities of the BoNTs are used for detection and serotype determination. Firstly, the toxin molecules in a biological sample are captured by serotype specific antibodies immobilized on magnetic beads and secondly, the toxin cleaves peptide-based serotype-specific substrates where after the cleavage products are identified by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). This approach yields two levels of serotype selectivity as well as an increased sensitivity compared to direct measurement of the BoNT, since each toxin molecule can catalyse the cleavage of numerous peptides, which amplifies the signal. For most BoNT serotypes, the detection sensitivity has been comparable or higher than that of the mouse bioassay in serum. In more complex matrices, like gastrointestinal contents, the detection of BoNT may be hampered by the presence of proteases yielding nonspecific cleavage products with variable sizes.

The endopeptidase reaction and the resulting cleavage products are highly specific for the activity of the light chain of the BoNT. However, other proteases present in the matrix (e.g. trypsin) may recognize

and cleave exactly at the same position resulting in false positive results (Wang et al., 2015, Wang et al., 2017, Kalb et al., 2019). In particular, the C and D light chain share their cleavage sites with trypsin which is encountered in a broad range of clinical matrices. Thus, extra care has to be taken to reduce the risk of this type of unspecific cleavage. The addition of protease inhibitors can help to prevent false positive and false negative results originating from matrix proteases (Wang et al., 2011), Tevell Åberg et al., 2018, Karlsson 2018).

8.2 Methods available for the detection of BoNT-producing clostridia

Whatever the sample, the strategy is to obtain a ten-fold dilution of the sample in an enrichment broth, and to incubate it under anaerobic conditions. Subsequently the presence of BoNT-producing clostridia, is demonstrated following DNA extraction and the implementation of a PCR targeting BoNT genes.

As there is no normative text for the detection of BoNT-producing clostridia in animal samples or for type C, D, C/D and D/C on the contrary to food samples, each laboratory has developed its own protocol for detecting BoNT-producing clostridia.

8.2.1 Enrichment broth

Several enrichment broths can be used for detecting BoNT-producing clostridia. Composition of media are provided in Appendix 2.

Table 1: Enrichment broths and sample processing for BoNT-producing clostridia detection

Broth	Matrix	Dilution	Incubation time	Incubation temperature	Reference
TPGY	Liver Ruminal content	Whole or 25g	At least 24 hours	37°C	(Le Marechal et al., 2017)
TPGY		1-2 g	24h-96h	30°C	(Anniballi et al., 2012)
F-CMM	Intestinal content, liver, ruminal content, faeces, environmental samples (feed, poultry litter, water, etc.)	1 g	48h	37°C	(Vanhomwegen et al., 2013, Bano et al., 2015, Bano et al., 2017)

TGY+CMM	Liver Feed Stomach content		5 days in CMM followed by 1 day in TGY	37°C	(Prevot et al., 2007)
TPGY or FCMM	Intestinal content, faeces, liver/spleen	1-2g	24h-96h	30°C	(Anniballi et al., 2013a)
Cooked-meat broth	intestinal contents, stomachs, spleens, and livers.	10 g	7 days	37°C (60°C-1h)	(Franciosa et al., 1996)

8.2.2 DNA extraction

Studies comparing DNA extraction kits have been conducted (Auricchio et al., 2013, Le Marechal et al., 2018); depending on the type of matrix being analysed (organs, water, feed, manures, etc.), different kits are used to optimize detection sensitivity.

By consensus, the DNeasy Blood &Tissue kit or the QiaAmp DNA mini-kit (Qiagen) are the kits that perform best (Auricchio et al., 2013, Le Marechal et al., 2018). DNA extraction methods based on Chelex beads are adequate for PCR diagnosis (Auricchio et al., 2013). Other kits, such as the Powersoil DNA isolation kit (Qiagen) and the Nucleospin Soil kit (Macherey), are, respectively, the most appropriate for the detection of *C. botulinum* in environmental and manure samples (Le Marechal et al., 2018).

8.2.3 PCR

Several PCRs have been developed and validated; ready-to-use PCRs (Woudstra et al., 2012), and multiplex PCRs, are available (Anniballi et al., 2012, Anniballi et al., 2013a). Primer sequences are provided in Appendix 3.

8.3 Sample collection, storage, and shipment recommendations

When feasible, samples should be obtained from live symptomatic animals or freshly euthanized symptomatic animals. *C. botulinum* is a bacterium that commonly grows on carcasses. It is challenging to determine whether *C. botulinum* or BoNT presence, is directly involved in the death of the animal, or is derived from its carcass as a post-mortem finding. However, when only samples from dead animals are available, an accurate diagnosis can be made taking into account the analytical results, the clinical signs, and epidemiological context. A brief description of the sampling conditions must be provided for all samples shipped to the laboratory conducting the analyses.

It is recommended to provide samples collected on several animals when feasible and not only one. For example, for the detection of BoNT-producing clostridia in birds using PCR, analysis of livers collected from at least four symptomatic animals is required to guarantee its detection (Le Marechal et al., 2016a).

Samples should not be pooled, in particular sera; these should be sent individually as pooling reduces the pathogen detection sensitivity. Serum, faeces, intestinal content, liver and other internal organs, feed can be analysed for BoNT detection using the Mouse bioassay, ELISA and Endopep-MS. The same samples except serum can be analysed for the detection of BoNT-producing clostridia using PCR, the Mouse bioassay, ELISA and Endopep-MS. For the detection of BoNT-producing clostridia, an enrichment step before any further analysis is required. Culture conditions (sample size, sample process, medium, incubation duration, temperature) vary according to the method used to confirm the presence of BoNT-producing clostridia, whether the method focuses on the detection of BoNT encoding genes of BoNTs.

Samples should be stored frozen before analysis to guarantee optimal detection of BoNTs or BoNT-producing clostridia (Le Marechal et al., 2017, Smart et al., 1987, Piazza et al., 2011).

8.4 BoNT type according to animal species

Outbreaks of avian botulism mostly involve type C/D (Anniballi et al., 2013a, Le Marechal et al., 2016a, Le Marechal et al., 2017, Woudstra et al., 2012, Bano et al., 2017). Some type D/C and D outbreaks have been diagnosed in turkeys (Le Marechal et al., 2016a, Le Marechal et al., 2017, Woudstra et al., 2012) and type D/C in stork (Bano et al., 2017). Type E outbreaks have also been reported both in poultry (AFSSA, 2002) and wild birds (Chun et al., 2013, Perez-Fuentetaja et al., 2011). Considering that type E is associated with cases of botulism in humans, the management of such outbreaks deserves specific attention.

Outbreaks of botulism in cattle involve type D/C and to a lesser extent type C (Bano et al., 2017, Woudstra et al., 2012). Type B outbreaks have been reported (Notermans et al., 1981, Yeruham et al., 2003) but are uncommon, at least in Europe.

Type B, C, D, and A outbreaks have been reported to occur in horses, mules or donkeys (Johnson et al., 2014a, Lanci et al., 2019, Johnson et al., 2014b). Type B seems to be more common in the USA while types C and D are more common in Europe (Gerber et al., 2006).

Outbreaks involving type C occur mostly in furred animals (Lindström et al., 2004).

9 Epidemiological data collection and sampling

Identification of the source of contamination is essential for outbreak management. Elimination of the source of contamination is of primary importance to interrupt the spread of botulism to healthy animals and its recurrence. During the epidemiological investigations, to determine the source of the outbreak, a diverse array of samples is collected and analysed for the presence of BoNTs or BoNT-producing clostridia:

- Feed or all ration components
- Litter
- Water (water pipelines, well water, etc.)
- Manure if present on the farm or in the neighbourhood (in particular poultry manure)

- Equipment used to feed animals or recently used in the vicinity of the animals
- Swabs or boot-swabs collected from any suspicious area
- Leftover feed from the mix-wagon or a swab from the mix-wagon

To identify the source of the outbreak and be sure that detection of BoNT or BoNT-producing clostridia is not due to cross-contamination, specific attention is required when sampling is performed (gloves have to be changed between samples for instance) and when feasible samples should be collected from areas non-exposed to symptomatic animals. For example, if litter is suspected, a sample should be collected from the storage area. Feed should be collected from the silo or non-exposed bag or storage building.

Samples should be stored frozen before analysis.

Detailed questionnaires developed during the ANIBOTNET project are available in Appendix 7.

10 Risks factors and sources of the outbreak

In poultry

In poultry, the contaminant source in outbreaks of botulism is seldom identified. In one, it was shown to be the litter (rice hulls) (Bano et al., 2013), in another cross-contamination was suspected to have occurred between broilers and turkeys (Souillard et al., 2019). Manure samples collected from the broilers were positive for *C. botulinum* type C/D without any botulism clinical signs and the outbreak occurred in the next turkey flock that was raised in the same house.

Some outbreaks, in particular in turkeys, occurred after the departure of females for slaughtering and outbreaks involving only males are the most encountered ones. The impact of thinning on the initiation of botulism outbreaks has however never been evaluated.

In cattle:

In cattle, the contaminant source in outbreaks of botulism is often identified:

- Poultry manure
- Animal carcass in feed and/or water
- Contaminated silage or polyethylene packaged hay bales

Acidification of feed (pH<4) can help in preventing the development of *C. botulinum* in feed (Driehuis et al., 2017).

11 Molecular epidemiology and strain tracking

11.1 Strain isolation

C. botulinum group III strains are ones most commonly encountered in animal botulism outbreaks. These strains are quite difficult to isolate mostly because the BoNT gene is encoded on a phage, which is unstable under laboratory conditions (Anza et al., 2014, Woudstra et al., 2012). Moreover, the isolation of *C. botulinum* group III strains is done by commencing using broth-enrichment cultures which often consist of a mixed bacterial flora; hence the lack of selective solid media impedes rapid and easy identification of *C. botulinum* group III strains.

11.1.1 Strain isolation on EYA and BAB2 media

A useful method for isolating strains is one based on broth-enrichment in fortified cooked meat medium (FCMM) and subsequent plating on egg yolk medium (EYA) and blood agar base (BAB) (See appendix 2 for medium composition) (Bano et al., 2017).

Samples should be inoculated in FCMM, then heated at 80°C for 15 min (Kurazono et al., 1985) or at 71°C for 10 min, thereafter rapidly cooled under running water (Bano et al., 2015) and then incubated for 48h under anaerobic conditions.

A 50 µL of PCR-positive FCMM is taken from the bottom of the tube and plated onto both EYA and BAB2 media. Both plates are incubated for 48h in an anaerobic cabinet (5 % hydrogen, 5 % carbon dioxide, 90 % nitrogen). On EYA plates, colonies may appear raised or flat, the margins smooth, or rough. They usually exhibit surface pearly-zone iridescence (lipase positive) when examined under oblique light. Besides the pearly-zone iridescence, colonies of group III strains are usually also surrounded by a wide area (2-4 mm) of yellow precipitate (lecithinase positive). Small lecithinase- and lipase-positive colonies are then collected and transferred to tubes containing 10 ml of FCMM. On BAB2, the colonies of group III strains are small and appear weakly haemolytic; these are selected and also cultured in tubes containing 10 ml of FCMM. After incubation (48h at 37°C under anaerobic conditions) the cultures are tested by PCR for BoNT presence (Bano et al., 2015). This method has led to the isolation of 81 BoNT-positive *C. botulinum* group III strains (Bano et al., 2017).

11.1.2 Strain isolation using InstaGene™ Matrix (Bio-Rad)

Another isolation method developed at ANSES consists of streaking the pellet obtained after DNA extraction, using InstaGene™ Matrix, on an EYA plate. This approach led to the isolation of 21 *C. novyi sensu lato* strains and 14 *C. botulinum* group III strains (Le Gratiet et al., 2020).

11.1.3 Strain isolation using immunomagnetic separation

Antibodies targeting type C and C/D spores are produced and biotinylated (Anza et al., 2014).

One gram of each sample is inoculated into 9 ml pre-reduced TPGY broth, heated for 15 min at 70 °C and incubated anaerobically at 37°C for two days. The overnight culture is washed and incubated with biotinylated polyclonal antibodies developed towards spores of type C and C/D strain. After several washing steps (see Anza et al. 2014 for detailed protocol), the bead suspensions are heated at 70°C for 10 min, streaked onto McClung Toabe agar and incubated anaerobically at 37°C for 2 days. Selected

lipase- and lecithinase-positive colonies are cultured overnight at 37°C in 9 ml TPGY broth and tested by PCR for detection of a BoNT gene (7.2.3) and a *C. novyi sensu lato* chromosomal marker(Anza et al., 2014). This method led to the isolation of 15 *C. novyi sensu lato* (Anza et al., 2014).

11.1.4 Storage of neuro-toxigenic strains

- Incubate neuro-toxigenic cultures coming from a single colony (isolated as described in the paragraphs 10.1.1-10.1.3) at suitable conditions for 2 days to up to 2 weeks, with the aim of increasing the number of spores.
- At the end of incubation time, centrifuge the culture and discard the liquid phase. If as enrichment broth FCMM was used, it is necessary discard the $CaCO_3$ and the meat particles residuals by washing the pellet using sterile water. With the aim of obtaining cells/spore free of carbonate and meat particles, several washing/centrifugation cycles may be necessary. Carbonate pellet is usually placed at the bottom of the tube and appears as milk white instead cells usually appears as off-white.
- Resuspend the pellet with the storage media or using the media contained in ready-to-use systems used for bacterial storage and retrieval such as Microbank™. As storage media it is possible to use TPGY containing 4% (w/w) glycerol or Tryptone Soya Broth (TSB) containing 0.5% glucose, 2% skim milk powder, 4% glycerol. Strains can also be stored in -80 ◦C in cultivation medium with 20% glycerol.
- Store the vials at -70°C or under nitrogen atmosphere. It is advisable renovate the culture stored in TPGY containing glycerol or TSB each 2-3 years. Strains stored in Microbank™ can be renovate each 9 years.

11.2 PCR-based subtyping methods

Considering the above-mentioned difficulties to isolate *C. botulinum* group III strains, the development of methods that do not involve strain isolation become important. PCR-based methods can achieve this goal. During the ANIBOTNET project, a Multilocus Variable-Number of Tandem-Repeat analysis (MLVA) method was successfully developed. MLVA is based on PCR analysis of tandem repeat sequences in the bacterial genome, in which specific primers are used to target the flanking sequences of variable number of tandem repeat regions. Since MLVA is a PCR-based method, only small amounts of target DNA are required. Standard operating procedure (SOP) for MLVA is available in appendix 4.

This method consists in the amplification of 9 different targets using PCR and subsequent determination of the number of repeats for each target. According to the number of repetitions for each target, the strain is assigned to a MLVA group.

Not only is this method useful for strain comparison but also can be used on DNA extracts in the absence of strain isolation (Auricchio et al., 2019).

Another method was developed within the ANIBOTNET project, namely, Multilocus Sequence Typing (MLST), but requires isolation of the strain. MLST consists in the sequencing of 14 different genetic

targets and then comparison of the sequence that will determine the sequence type (ST) of the strain. SOP for MLST is presented in appendix 5.

Compared to MLST, the higher resolution ability of MLVA reveals it to be the more appropriate method for typing *C. botulinum* group III strains (Scalfaro et al., 2019).

11.3 Other subtyping methods

11.3.1 Pulsed-field gel electrophoresis (PFGE)

PFGE is a classical typing method commonly used for bacterial strain comparison and tracking. A fully detailed protocol is available (Skarin et al., 2010). This method was used to comparison strains isolated during avian botulism outbreaks in Spain and in Scandinavia and showed a high similarity between strains (Anza et al., 2014).

11.3.2 Randomly amplified polymorphic DNA analysis (RAPD)

RAPD is a classical typing method commonly used for bacterial strain comparison and tracking. A fully detailed protocol is available (Skarin et al., 2010). The RAPD system is more difficult to interpret than the PFGE system. However, RAPD can be used as a complement to PFGE for pulsed-field gel electrophoresis untypable strains and also for primary screening because the results can be obtained much faster (Skarin et al., 2010).

11.3.3 Amplified fragment length polymorphism (AFLP)

AFLP method has also been tested for the comparison of all *C. botulinum* strains and a fully detailed protocol is available (Hill et al., 2007). This method allows the clustering of *C. botulinum* group III strains according to BoNT type (C or C/D), the source of isolation, as well as to geographic location (Hill et al., 2007). This was used to investigate the source of contamination during an outbreak of bovine botulism (Myllykoski et al., 2009).

11.3.4 Matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

A MALDI-TOF MS approach has been used to compare and phylogenetically cluster *C. botulinum* group III strains. Using this method, it is possible to cluster *C. botulinum* group III according to BoNT type. A detailed protocol is available (Bano et al., 2017).

11.3.5 Whole Genome Sequencing (WGS)

Sequencing of the whole genome is about to become the reference method for bacterial typing. Having whole genome data allows for highly discriminant strain comparisons to be made and is the most powerful tool available for tracking strains. At present, approximately forty *C. botulinum* group III genomes are available in public databases (Woudstra et al., 2016). SOP for DNA extraction to perform WGS is presented in appendix 6.

11.3.6 Mass Spectrometric Protein Sequencing of BoNTs

The BoNT subtype can be determined after antibody trapping of the toxin, tryptic digestion and mass spectrometric analysis of the resulting peptide fragments. The masses of the resulting peptide fragments can be compared to a database and variations in amino acid composition and thus BoNT

subtype information can be obtained. A protocol for this procedure has been developed during the project.

12 Outbreak management of farm products

In the event of an animal botulism outbreak, risk evaluation should be considered for in-farm products of animal origin (milk and milk products, eggs and egg products, and meat). The restrictions, regulations and conditions, that should be applied to determine whether the products are suitable for human consumption, are not well defined yet.

Regulation (EC) No 854/2004 of the European parliament and of the council of 29 April 2004 indicates that « […] » animals showing clinical signs of systemic disease or emaciation, are not to be slaughtered for human consumption. Such animals must be killed separately, under conditions such that other animals or carcasses cannot be contaminated and declared unfit for human consumption».

The main questions to be asked are:

- Can eggs be sold during and after a botulism outbreak in a poultry flock? Under which conditions?
- Can milk be collected during and after an outbreak of botulism? Are some risky processes involved?
- Can asymptomatic animals from botulism-affected farms be harvested, and are there specific procedures that should be avoided?

12.1 Human cases linked to animal botulism

Very few human cases have been linked to outbreaks of animal botulism. No clinical disease symptoms have been reported in farmers, veterinarians or people charged with the disposal of carcasses during outbreaks.

A case of human botulism is suspected to have been linked to the consumption of poultry obtained from a botulism type C-positive flock; however, no BoNT C was detected in samples obtained from the patient (Martrenchar et al., 2019).

As mentioned earlier, botulism in humans is mostly associated with BoNT types A, B, E and F, while in animals mostly BoNT types C, D, C/D and D/C are involved. Animals and animal products from farms where the presence of BoNT type A, B, E or F is confirmed should not be consumed by humans and carcasses should be destroyed (AFSSA, 2002).

With regards to human health, the management of outbreaks involving types C, D, C/D and D/C, is more complicated, because the zoonotic potential of these BoNT types has not yet been fully clarified. Worldwide, only 11 human cases (involving 6 outbreaks) have been reported.

12.2 Milk

It is unknown if BoNT is secreted in milk by cattle suffering from botulism (Lindström et al., 2010). In only one study has BoNT been detected in the milk of rats suffering intestinal botulism (Moberg and Sugiyama, 1980). To the contrary, BoNT type C was not detected in milk inoculated into the udder vein of a dairy cow (Moeller et al., 2003). Cobb et al. have concluded from their study that it seems unlikely

that BoNT is secreted in milk (Cobb et al., 2002). In a *C. botulinum* type C outbreak in cattle, using MBA, samples of tank milk were found to be BoNT negative (Bano, 2019).

Reports suggest that HTST pasteurization (72°C/162°F for 15 seconds) is likely to inactivate most or all of the BoNT toxins that may contaminate milk (Weingart et al., 2010); conventional pasteurization at 63°C/145°F for 30 min. seems to be less effective (The center for Food Security and Public Health, 2018).

It is advised that milk from animals showing symptoms of disease, should not enter the human food chain. The UK Food Standards Agency recommends a wait of 14 days, following the last case of botulism, before the milk can be used again safely for human consumption (Lindström et al., 2010). A delay of 15 days is also recommended in a French report (AFSSA, 2002). Outbreaks in cattle are reported to be biphasic, comprising an acute and rapid phase followed by a delayed phase; clinical signs manifest in the first few days, with the delayed phase occurring around 2 weeks later, following first exposure to the contaminant source (Myllykoski et al., 2009, Neill et al., 1989, Relun et al., 2017). This biphasic disease pattern explains the recommendations involving safe milk consumption.

While the risks to human health posed by milk from farms affected by botulism are considered to be negligible, weak or non-existent depending on how the milk is treated (Souillard et al., 2016), the risk to develop infant botulism, even with regard to the less-pathogenic C, D, C/D and D/C BoNT strains, is unclear.

12.3 Meat

As mentioned earlier, regardless of the BoNT type involved, animals showing clinical signs should not enter the food chain. Regarding animals showing with no clinical signs on farms experiencing an outbreak, the risk to human health is considered, null or negligible depending on how the meat was processed (AFSSA, 2002).

12.4 Eggs

In an outbreak of botulism on a poultry farm, only laying hen shells, not the contents of the egg, may be contaminated (Souillard et al., 2016). The risk of eggs and egg products to human health is considered negligible or non-existent (AFSSA, 2002).

13 Therapeutic measures

The prognosis depends on the clinical stage of the disease. Recovery has been reported in animals with mild clinical signs (Kummel et al., 2012, Prevot et al., 1955, Le Maréchal et al., 2019, Lindström et al., 2004, Bano et al., 2015). In acute cases, death is caused by respiratory paralysis (Kummel et al., 2012).

13.1 In poultry

Beta-lactam antibiotics added to drinking water are effective at halting clinical signs and mortality in botulism affected poultry flocks. However, respecting drug withdrawal periods (compulsory before harvesting) often results in the recurrence of clinical signs. Separating healthy animals from those that are affected, the addition of fresh uncontaminated litter, combined with beta-lactam antibiotic treatment, can be helpful in managing and controlling the outbreak. Treatment of botulism is usually not successful in the advanced stages of the disease and euthanasia is frequently advised (Bano, 2019). To prevent recontamination of the flock, the removal of contaminated litter, if feasible, or the addition of a fresh layer, can be helpful for controlling and containing the outbreak. Daily removal of carcasses during the outbreak is highly recommended to prevent the development of maggots that may ingest and concentrate BoNT and BoNT-producing clostridia.

13.2 In wild birds

When possible, affected waterfowl should be provided with fresh water and shade or treated with antitoxins; recovery rates of 75-90 % have been reported. Treatment of shorebirds, gulls, American coots, and grebes are usually not successful. Recommended control measures include removal of bird carcasses, discouraging birds from using contaminated areas (The center for Food Security and Public Health, 2018), and modification of the environment, such as decreasing water temperature, remove mud or oxygenase water (Son et al., 2018).

13.3 In cattle

Emergency vaccination is commonly used in cattle during outbreaks to protect animals that do not show clinical signs (Le Maréchal et al., 2019, Souillard et al., 2017); this strategy is effective in controlling outbreaks (Bano, 2019). Vaccination is in particular recommended when the source of BoNT cannot or has not been identified. In France, Ultravac® Botulinum (Zoetis) can be used only under 'temporary use' authorization. A list of commercial vaccines is available (Anniballi et al., 2013b).
Antitoxins can be administered to affected animals but is effective only when the toxin is in circulation (Bano, 2019, Guizelini et al., 2019).

13.4 In horses

The treatment of botulism in horses relies on supportive care and antitoxin treatment (Galey, 2001); Antibiotic treatment is administered, especially in case of wound botulism (Galey, 2001). The survival rate is low in horses exhibiting advanced clinical signs (from 10 % in BoNT type A outbreaks, to 30 % involving BoNT type B). The survival rates for hospitalized foals are as high as 96% (Johnson et al., 2014a); horses unable to stand have a poor chance of survival (Johnson et al., 2014a). Mechanical

ventilation of a recumbent horse, combined with the administration of a pentavalent antitoxin, was successful in an adult horse (Taylor et al., 2014); mechanical ventilation reduces also the death rate in foals (The center for Food Security and Public Health, 2018).

14 Sanitation

14.1 Cleaning and disinfection.

Based on an estimated toxin decay rate of 1%-4%/min, BoNTs are inactivated within 1-3h following exposure to sunlight (The center for Food Security and Public Health, 2018, Villar et al., 2006); the decay rate however is dependent on weather conditions (e.g., temperature, humidity) and on the dispersal pattern of the toxin within the environment (Villar et al., 2006).

Thermal treatment (80°C for 20 min or more than 85°C for 5 minutes) is efficient for BoNT eradication. BoNTs also can be eliminated by applying 0.1% sodium hypochlorite or 0.1N NaOH, while the addition of chlorine helps to eliminate waterborne BoNTs (The center for Food Security and Public Health, 2018).

Clostridium botulinum is highly resistant and its ability to persist in the environment over an extended period of time is well documented (Notermans et al., 1981, Smith et al., 1975, Wobeser et al., 1987). Following an outbreak involving 37 farms, 26.4% of the samples obtained from poultry houses and the immediate surroundings, were *C. botulinum* positive; amongst these, swabs from boots used to walk on the litter, were the greatest source of *C. botulinum* positive samples (48.1 %) (Souillard et al., 2018). Following thorough disinfection, the fact that 13 % of the samples obtained from 26 farms were positive, revealed that *C. botulinum* still persisted.

Darkling beetles, drinking water, the ventilation system, the surrounding soils, and the poultry houses, are the critical areas that may remain contaminated following an outbreak (Souillard et al., 2014). Appropriate cleaning and disinfection operations are necessary to eradicate *C. botulinum* spores and thereby prevent the recurrence of the disease. It is noteworthy that presence of *C. botulinum* spores in the environment of a farm after an outbreak does not automatically result in a new outbreak (Okamoto et al., 1999) but represents latent risk, bearing in mind that the disease often recurs in poultry houses previously infected.

A strengthened disinfection protocol must, firstly, be implemented and, second, may need to be adapted according to specific farm practices. The critical contaminated areas are listed above. Material infrastructure (e.g., feeding troughs) must be disassembled, thoroughly cleaned, and then disinfected. All forms of organic matter, in particular litter, must be removed and eliminated. A first step dedicated to cleaning is of main importance so as to get rid of dust and organic matter and is crucial to be able to efficiently disinfect in a second step. Thereafter, the premises must be disinfected using sporicidal agents. There are only a few disinfectants effective for the complete elimination of *C. botulinum* spores. These include the following recommendations:

- Sodium-hypochlorite:
    - A 10% solution of sodium-hypochlorite for 20 minutes (Bano, 2019);

  - 0.1% (1000 ppm) sodium-hypochlorite for 5 minutes (Oie et al., 2011)
  - 0.26% (2600 ppm) sodium-hypochlorite for 15 minutes (Souillard et al., 2018)
- Per-acetic acid:
  - A 3% solution of per-acetic acid for 10 minutes (Bano, 2019).
  - 0.3% peracetic acid for 5 minutes (Oie et al., 2011)
- 2% glutaral for 6 hours (Oie et al., 2011)
- Hydrogen peroxide vapour (Johnston et al., 2005)
- 5 % hydrogen peroxide for 15 minutes (Souillard et al., 2018)

Ethylene, propylene oxide and formaldehyde are all able to inactivate *C. botulinum* spores, but not very rapidly (Smith, 1977). Despite its high toxicity, formaldehyde is still sometimes used during outbreaks of botulism. Most of the disinfectants listed above are highly toxic, and their safe handling necessitating the use of dedicated protective equipment.

Assessing the effectiveness of the disinfection program can be done by follow-up swabbing of the critical areas known to be difficult to decontaminate. These swabs are then diluted in 250 mL TPGY, incubated under anaerobic conditions for 4 days; thereafter, detection of BoNT genes is attempted using PCR following DNA extraction. This is necessary to determine whether additional disinfection operations are required before the animal premises can be freshly restocked.

Using quick lime, outdoor free-range areas are decontaminated to prevent recurrence of the disease. However, despite residual contamination in laying hen farms, no other outbreaks were recorded (Souillard et al., 2016, De Santis et al., 2019). In a free-range farm of pheasant, where the wide extension of land does not allow an accurate disinfection, sporadic cases of botulism occur annually (De Santis et al., 2019)

14.2 Management of manure after an outbreak.

Consequent to an outbreak, farm manure will be left contaminated and will remain so for several months thereafter (Le Maréchal et al., 2017, Notermans et al., 1981). Contaminated manure if left *in situ* can be the source of a new outbreak (Souillard et al., 2017); in particular, contaminated poultry manure is of great risk to cattle (Payne et al., 2011, Relun et al., 2017, Souillard et al., 2017).

By spreading contaminated manure the pastures will also become contaminated (Notermans et al., 1981). In this context, it has been demonstrated that *C. botulinum* spores can persist for up to 939 days (study length) in soils fertilized with spore-spiked compost (Gessler and Bohnel, 2006).

As far as public and animal health is concerned, European regulation describes the management of animal by-products that include manure (Regulation (EC) No 1069/2009). According to this regulation, it is legal to spread manure over land without any prior treatment if the competent authority considers that there is no risk of contamination. If there is a known risk of contamination, the manure has to be destroyed. In the event of an outbreak of any notifiable animal disease, when transporting manure to the nearest burning plant increases the danger of microbial propagation, the competent authority may, by derogation, authorise its disposal by burning or burial on site under conditions that prevent risks to public and animal health.

Three methods are used to dispose of contaminated manure: burning, lime-treatment, and incineration. Of the three, the burning of manure in the open, though not commonly applied, must be conducted with care and preferably under the supervision of qualified firemen to avoid the initiation of an uncontrollable fire and the excessive emission of smoke into the local environment (AFSSA, 2002, SciCom, 2017). *C. botulinum* has not been detected in the ashes of burned manure (Le Maréchal et al., 2019). Alternatively, to promote exothermic combustion, 5%-7% lime (calcium hydroxide, $Ca(OH)_2$) can be added to contaminated manure (Balloy et al., 2009). The aim of treatment with lime is to reduce the quantity of manure handled for burning; however, it has been shown that lime-treated manure may still remain *C. botulinum*-positive (Le Maréchal et al., 2017). The third method involves the use of an incineration plant; however, incineration is expensive and the transport of contaminated manure from the farm to the plant may cause a widening in the spore dissemination area. Furthermore, the decontamination of the equipment used to transport the manure has also to be undertaken.

The thermophilic anaerobic digestion of *C. botulinum* (types C, D, C/D or D/C) contaminated manure can be achieved using a biogas plant, but only if the manure is pre-treated at 70°C for 1 h (SciCom, 2017). The alternative use of mesophilic anaerobic digestion (i.e., without heat pretreatment at 70°C for 1h) is the type of agricultural biogas plant most commonly found on French farms for example, but does not ensure definitive eradication of *C. botulinum* (Neuhaus et al., 2015).

In the final analysis, there is no optimal solution available today for the handling of *C. botulinum*-contaminated manure. It is known that after an outbreak manure is contaminated, that this contamination is persistent and that it constitutes a potential source for a new outbreak of botulism. Thus, the eradication of *C. botulinum* is essential and, currently, remains the only solution available to the farming community and health authorities. Manure must be carefully managed to avoid cross-contamination between productions and recurrence of the disease on the same farm.

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16 Appendix 1

Table 2 : List of references where pictures illustrating clinical signs or detailed description of clinical signs are available

Animal species	Reference
Cattle	(Dlabola et al., 2016, Guizelini et al., 2019, Le Maréchal et al., 2019, Le Marechal et al., 2016b, Myllykoski et al., 2009, Mariano et al., 2019)
Poultry Laying hens	(AFSSA, 2002, Skarin et al., 2015, Le Marechal et al., 2016b)
Wild birds	(Le Maréchal and Souillard, 2013, Rocke and Bollinger, 2007)
Fur animals	(Gustavsen et al., 1969)
Fishes	(Eklund et al., 1984, Eklund et al., 2004)
Pigs	(Raymundo et al., 2012)

17 Appendix 2: Composition of broth media for BoNT producing clostridia culture

TPGY (Skarin et al., 2010) Tryptose_peptone_glucose_yeast extract

This medium should be boiled 15 minutes before use. It is recommended for detection of *C. botulinum* in field samples or strain culture.

5% Tryptone (Difco),

0.5% Proteose Peptone (Difco)

0.4% glucose

2% yeast extract (Oxoid)

0.1% starch (Merck)

Supplemented with 0.1% l-cysteine_HCl and 0.14% $NaHCO_3$

F-CMM

For 1000 ml: Bacto Proteose peptone (Oxoid) 20 g

Yeast extract (Oxoid) 10g

$(NH_4)_2SO_4$ 10g

NaCl 5g

Starch 5 g

Glucose D+ 10 g

L-Cysteine HCl 1 g

$CaCO_3$ 5g

Cooked meat medium (Oxoid) 1g/tube of 9 mL

McClung Toabe Agar plate (Skarin et al., 2010)

4% Proteose Peptone (Difco)

0.2% NaCl

0.2% glucose

0.5% $NaHPO_4$

0.1% KH_2PO_4

0.02% $MgSO_4$

0.5% yeast extract (Difco)

2.5% Bacto agar (Difco)

supplemented with 0.1% l-cysteine_HCl and 0.14% NaHCO3

EYA (Bano et al., 2015)

Blood Agar Base No.2 (Oxoid) as a base

50 ml/L yolk solution (25 ml fresh yolk and 25 ml of saline solution)

BAB2 (Bano et al., 2015)

Blood Agar Base No.2 (Oxoid) as a base

5% defibrinated sheep blood

2.5% agarose

18 Appendix 3: PCR primers and conditions

(Anniballi et al., 2012)

Target	gene	Primer														Position	GeneBank	acc.no.
bont/C	FABO_c_F	gag	Cct	gaa	aaa	gcc	ttt	cgc	a							108	239–260	D49440
	FABO_c_R	tag	Ggc	ttg	taa	ctc	gag	gag	gtt							324–347		
bont/D	FABO_d_F	tta	Tgg	gag	att	caa	gta	cgc	ct							422	509–531	D38442
	FABO_d_R	atc	Cct	cgc	taa	ctt	gtg	gac	gaa							908–931		
IAC	FABO_iac_F	tta	Tgg	gag	att	caa	gta	cgc	ctT	GAT	GAG	CAT	CAC	AAA	AAT	CGa	153+46b	888–1008	L09137
	FABO_iac_R	atc	Cct	cgc	taa	ctt	gtg	gac	gaa	GAA	GGG	AGA	AAG	GCG	GAC	AGa	1021–1040		

PCR conditions:

Total volume: 25µL:

- 3µL of template DNA
- 12.5 µL of 2X QuantiTect SYBR Green PCR Master Mix (Qiagen)
- 600 nM of each primer
- 3µL of IAC (150 copies/well).

95°C for 15 min, followed 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and followed by a melting curve analysis.

(Anniballi et al., 2013a)

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Primer and Probe sets

Target	Primer/probe	Sequences (5'-3')*	Position	GeneBank acc. No.
bont/C	Forward	cta cgt tta aat taa cta ac	76176-76195	AP008983
	Reverse	ggt gat att gaa att att g	76233-76251	
	Probe	cacCaaAtcCttCttgtg	76209-76226	
bont/D	Forward	cag gaa att ttg ttg taa	8745-8762	AB745669
	Reverse	cga aga ata aac aac ttc	8813-8830	
	Probe	acaTaaCatTagTcaagtct	8791-8810	
bont/CD	Forward	aca gga tat aca aat aaa tg	2998-3017	FN436022
	Reverse	cct cat cta aat ctt caa	3098-3115	
	Probe	ttcActCtgCttTaattct	3075-3093	
bont/DC	Forward	gtt cgt tta taa tac aac c	3620-3638	EF378947
	Reverse	cca agt ttg aaa cta taa c	3725-3743	
	Probe	tgaTagTatTccAagccta	3699-3717	
PC	Forward	agc agt tat ttc agt aaa	5396-5413	CP000939

	Reverse	cac cat ttc cta att ttg	5449-5466	
	Probe	tgtCtgTccTtcAaattg	5427-5444	

* bases written in Capital letters in probe sequences represent LNA modifications

Primer and Probe concentration and reporter labelling

Target	**Primer/ probe**	**Mx3005P**				**CFX96**			
		3-plex		**5-plex**		**3-plex**		**5-plex**	
		Conc (nM)	**Rep.**	**Conc (nM)**	**Rep.**	**Conc (nM)**	**Rep.**	**Conc (nM)**	**Rep.**
bont/C	Forward	600	-	600	-	300	-	300	-
	Reverse	600	-	600	-	300	-	300	-
	Probe	100	HEX	100	HEX	100	HEX	100	HEX
bont/D	Forward	600	-	600	-	300	-	300	-
	Reverse	600	-	600	-	300	-	300	-
	Probe	100	FAM	100	FAM	100	FAM	100	FAM
bont/CD	Forward	600	-	600	-	300	-	300	-
	Reverse	600	-	600	-	300	-	300	-

	Probe	100	HEX	100	Cy5	100	HEX	200	Cy5
bont/DC	Forward	600	-	600	-	300	-	300	-
	Reverse	600	-	600	-	300	-	300	-
	Probe	100	FAM	300	Alexa350	100	FAM	200	Cy5.5
PC	Forward	600	-	600	-	200	-	200	-
	Reverse	600	-	600	-	200	-	200	-
	Probe	50	ROX	50	ROX	50	ROX	50	ROX

Total volume: 25µL:

- 3µL of template DNA
- 12.5 µL of 2X QuantiTect Multuplex PCR no ROX kit (Qiagen)
- Primers and probe concentration as described the table above

95°C for 15 min, followed 40 cycles at 94°C for 30 s, 56°C for 90 s.

(Woudstra et al., 2012)

Primer or probe	sequence	Target BoNT		position	size
C-I_F	TCCTGGGAATAACAATACTC	C	C-D	348–367	135

C-I_R	CTAGGTCCAGTTATTATAACAC			485–464	
C-I_P	[ROX]AACCCAGTTGTTACCTTGTCTAGTTT[BHQ2]			441–416	
C-II_F	GGGTCAAAATTTATCTCG	C	C-D	1233–1250	135
C-II_R	AGCTCTCTACAATCTAATG			1367–1349	
C-II_P	[ROX]ATCCAGCATTAAGAAAAGTCAATCCT[BHQ2]			1253–1278	
C-III_F	TCAGCTTAATCCAATATTTCC	C	D-C	2697–2717	77
C-III_R	GGGTTACTATAACTTTACCTC			2773–2753	
C-III_P	[ROX]CCCTGAACTACCTAATTTAAAGTCAAA[BHQ2]			2745–2719	
D-C_F	GACTGATTTAGTTCCACTAG	D-C		3561–3580	82
D-C_R	GCATGGTTGTATTATAAACG			3642–3623	
D-C_P	[ROX]ACGTATCTCATCCATTGGTTGATC[BHQ2]			3612–3589	
D-I_F	TCCAGTAATAGCTTTAATGC	D	D-C	666–685	141
D-I_R	TCCTCAAATTGTACGTTG			806–789	
D-I_P	[ROX]AAATCCCTCGCTAACTTGTGGAC[BHQ2]			771–749	
D-II_F	GAAGCATTAGGTTATAGCAATAAG	D	D-C	1696–1719	116
D-II_R	TCCTCAACTACTTCATTCG			1811–1793	
D-II_P	[ROX]AGAATAAACCTGCTTGAACACCTTT[BHQ2]			1783–1759	
D-III_F	ATGGCAATATAGAATGGA	D	C-D	2949–2966	138
D-III_R	ACCCCATTATATTATTAGTTATAG			3086–3063	
D-III_P	[ROX]ATCCTGTATGACTTAATGATTCACT[BHQ2]			3038–3014	

(Le Maréchal et al., 2019)

Type C :

P1652 (50-GGCACAAGAAGGATTTGGTG-30)

P1653 (50-TTGGATCCATGCAAAATTCA-30
Total volume of 25 µl :12.5 µl of 2Xconcentration of iQ SYBR green Supermix (Biorad),
5 pmole of each primer, 5 µl of template DNA and 7.3 µl of
1 cycle of 95°C for 10 minutes,
40 cycles of
95°C for 15 sec
60°C for 30 sec
1 cycle at 95°C for60 sec, 60°C for 60 sec and 95°C for 60 sec, melt curve

19 Appendix 4: MLVA SOPs

APPARATUS AND EQUIPMENT

- ✓ Capillary electrophoresis apparatus (Agilent Bioanalyzer 2100).
- ✓ Centrifuge tube, of capacities of 15 ml.
- ✓ DNA Sanger sequencer.
- ✓ Graduated pipettes, capable of taking volumes between 1 and 10 ml.
- ✓ Micro-centrifuge tubes, of capacities of 1.5 ml and 2.0 ml.
- ✓ Micro-centrifuge, for 2.0 ml tubes and with adjustable acceleration of up to 14 000 x *g*.
- ✓ Thin-walled PCR microtubes, 0.2 ml reaction tubes or multi-well PCR microplates.
- ✓ Thermal cycler.
- ✓ Pipette filter tips, for volumes between 1µl and 1 000 µl.
- ✓ Variable volume micropipettes, capable of taking volumes between 1µl and 1 000 µl.
- ✓ Bionumerics software for VNTRs analysis.

REAGENTS

- ✓ Nuclease-free water.
- ✓ Primer set for each VNTRs.
- ✓ Qiagen HotStarTaq Master mix kit (Cat. 203443) or Qiagen Multiplex PCR kit (Cat.206143).
- ✓ Reagent for PCR-product sizing (Agilent DNA 7500 kit cat. 5067-1506 or DNA12000 kit cat 5067-1508).

LIST OF PRIMERS

N.	Name	Left Primer	Name	Right Primer
14	MLVA_ANB_14L	CATATTTCCGCAGCCGTT*	MLVA_ANB_14R	TGTGTTGAAGGCATGAATCC
15	MLVA_ANB_15L	TGGATCATGTGAAAATGCAAG	MLVA_ANB_15R	CAGCTCCTGCCTTCGCTA
16	MLVA_ANB_16L	GAAGCTGTAGGGCGTAGACAA	MLVA_ANB_16R	TGTCCTGGCATCAATTCTGA
17	MLVA_ANB_17L	TTTCTTCGTGCAATCCCTCT	MLVA_ANB_17R	GAAAATGCAAGGATGCTAAGA
18	MLVA_ANB_18L	TGTGAACCTGCCAACCCT	MLVA_ANB_18R	GGTGCACATTGACAACAGC
19	MLVA_ANB_19L	TTATCCGGGGTTCCCATT	MLVA_ANB_19R	CAACTTGATACCGGCCCC
20	MLVA_ANB_20L	GGACGCAACCTCAGAGAATG	MLVA_ANB_20R	ACTTCTTCGTCCATAGCTCCA
23	MLVA_ANB_23L	GGTTTAGATGCTAGAGGGTTTGA	MLVA_ANB_23R	AACGTGCTTCAAAAGGCAGT
24	MLVA_ANB_24L	CTGTTTCTCCCCTTTGTGTCA	MLVA_ANB_24R	GGCGTTAAAGGAAGCGGTAG

PROCEDURE

- ✓ Extract DNA according to the specific SOP (Standard Operating Procedure on DNA extraction from *Clostridium botulinum* group III cultures).
- ✓ Prepare the master mix as following described, considering a volume 10% greater than that required, to compensate for pipette calibration.

Component	Volume/reaction
HotStarTaq master mix*	12.5 µl
Left primer (10 mM) - final concentration 500 nM	0.125 µl
Right primer (10 mM) - final concentration 500 nM	0.125 µl
Template DNA	3 µl
Nuclease-free water	9.25 µl
TOTAL VOLUME	25 µl

*the protocol was optimized also using Multiplex PCR kit Qiagen.

- ✓ Dispense the master mix in the PCR wells.
- ✓ Dispense the DNA in each well.
- ✓ Run the PCR according to the following thermal profile.

Phase	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling		
denaturation	30 sec	94°C
annealing	60 sec	55°C
extension	60 sec	72°C
Number of cycles 35		

Final extension	5 min	72°C

- ✓ Size PCR product as described in the procedure reported in Appendix 1.
- ✓ Extract the VNTR (allele) for PCR product size using the Excel macro.
- ✓ Include the MLVA profile in the project database or analyse the data using Bionumerics or other suitable software.

WARMING

If you during the PCR product sizing process you find new alleles, these need to be confirmed by means of DNA Sanger sequencer sizing and sequencing. The present procedure does not describe the sizing and sequencing by means of DNA Sanger sequencer. Please carry out sizing and sequencing using the procedures described by the DNA Sanger sequencer producer.

20 Appendix 5: MLST SOPs

APPARATUS AND EQUIPMENT

- ✓ Bionumerics software for sequences analysis and clustering.
- ✓ Centrifuge tube, of capacities of 15 ml.
- ✓ Cuvettes for DNA quantification.
- ✓ DNA Sanger sequencer.
- ✓ Graduated pipettes, capable of taking volumes between 1 and 10 ml.
- ✓ Micro-centrifuge tubes, of capacities of 1.5 ml and 2.0 ml.
- ✓ Micro-centrifuge, for 2.0 ml tubes and with adjustable acceleration of up to 14 000 x *g*.
- ✓ Pipette filter tips, for volumes between 1µl and 1 000 µl.
- ✓ Spectrophotometer for DNA quantification.
- ✓ Thermal cycler.
- ✓ Thin-walled PCR microtubes, 0.2 ml reaction tubes or multi-well PCR microplates.
- ✓ Variable volume micropipettes, capable of taking volumes between 1µl and 1 000 µl.
- ✓

REAGENTS

- ✓ Nuclease-free water.
- ✓ Primer set for each target gene.
- ✓ Thermo Scientific Master mix Phusion Hot Start DNA polymerase (cat. F-549L).
- ✓ Qiagen QIAquick PCR purification kit (cat. 28104)
- ✓ Reagent for PCR-product sizing (Agilent DNA 7500 kit cat. 5067-1506 or DNA12000 kit cat 5067-1508).
- ✓ Cycle sequencing reagents.
- ✓ Sequences purification reagents.

LIST OF PRIMERS

Gene	Name	Left Primer (5'-3')	Name	Right Primer (5'-3')	PCR product
adk	MLST_ABN_1L	GAATGATTTTGTTAGGACCTCC	MLST_ABN_1R	AATTGATTGTGTACCATCAACC	599
atpD	MLST_ABN_2L	CATAGACCAGCMCCAACTT	MLST_ABN_2R	TTAAGTCRTCHGCWGGWACA	583
dnaN	MLST_ABN_3L	CTGGMAAATCWCCWATGCC	MLST_ABN_3R	TSWARTTCRYCHCCTTGCA	877
efTu	MLST_ABN_4L	ACAACAACAACAGCAGCAAT	MLST_ABN_4R	TCCTGTTGCAACTGTTCCTC	612
GroEL	MLST_ABN_5L	GAATCAAGACGTGCTATGCA	MLST_ABN_5R	GSATTTTCTARTRCAGCTTCCA	620
gyrB	MLST_ABN_6L	GCHATGATWACAGCTTTYGGA	MLST_ABN_6R	ADAYTTCRTCRGCMCKCAT	418

lepA	MLST_ABN_7L	ACHACWGCKCCATCAGTT	MLST_ABN_7R	YTTWGCYCCTACRGCTGC	516
pheS	MLST_ABN_8L	THTCAARYAARGCAGAWCTTGA	MLST_ABN_8R	CGATAAACTTTTCCTGGYGCTA	532
proC	MLST_ABN_9L	GATTTATCGGATGTGGMAACAT	MLST_ABN_9R	CRTCYTTTAAHGCWCCWGGAT	666
rpL10	MLST_ABN_10L	GGTTAYGAWGATRCAACWGCA	MLST_ABN_10R	GGAGCTTTTAGGCTGCCA	185
rpoB	MLST_ABN_11L	TGGGATTCATAACTTGGGAAGG	MLST_ABN_11R	ACCTGACCGATATTCATACGAG	637
secD	MLST_ABN_12L	AAAGGHTTTGCMYTWACKCTT	MLST_ABN_12R	CCCTTTTRAYRCCAAAWTGGGA	136
secF	MLST_ABN_13L	AAARTAYGATMMWRCAGCKTCA	MLST_ABN_13R	ARTCTCTAACWGMHGGYACAA	569
spoA	MLST_ABN_14L	GATGARCTTCCWCARAGTGC	MLST_ABN_14R	ARTTCTGGWGAYACAAGTCCA	947

PROCEDURE

- ✓ Extract DNA according to the specific SOP (Standard Operating Procedure on DNA extraction from *Clostridium botulinum* group III cultures).
- ✓ Prepare the master mix as following described, considering a volume 10% greater than that required, to compensate for pipette calibration.

Component	Volume/reaction
Buffer 5X	10 µl
dNTPs 10 mM	1+1+1+1 µl
Taq Polymerase	0.5 µl
Left primer (100 mM) - final concentration 600 nM	0.3 µl
Right primer (100 mM) - final concentration 600 nM	0.3 µl
Template DNA	2 µl
Nuclease-free water	32 µl
TOTAL VOLUME	50 µl

- ✓ Dispense the master mix in the PCR wells.
- ✓ Dispense the DNA in each well.

✓ Run the PCR according to the following thermal profile.

Phase	Time	Temperature
Initial activation step	30 sec	98°C
3-step cycling		
denaturation	10 sec	98°C
annealing	30 sec	50°C
extension	30 sec	72°C
Number of cycles 35		
Final extension	5 min	72°C

✓ Purify the PCR product as described in Appendix 1.
✓ Prepare the master mix for cycles sequencing.
✓ Purify the sequence.
✓ Run sequence in DNA Sanger sequencer.
✓ Include the MLST profile in the project database or analyse the data using Bionumerics or other suitable software.

21 Appendix 6: SOPs for DNA extraction for WGS

APPARATUS AND EQUIPMENT

- ✓ Anaerobic cabinet or anaerobic jar, capable of being maintained at 37°C ±1°C.
- ✓ Autoclave, for buffers sterilization at 121°C and for infected material sterilization at 134°C.
- ✓ Centrifuge tube, of capacities of 15 ml.
- ✓ Centrifuge, for 15 ml tubes and with adjustable acceleration of up to 8 000 x *g*.
- ✓ Graduated pipettes, capable of taking volumes between 1 and 10 ml.
- ✓ Incubator, capable of operating at 37°C ±1°C.
- ✓ Inoculating loops.
- ✓ Micro-centrifuge tubes, of capacities of 1.5 ml and 2.0 ml.
- ✓ Micro-centrifuge, for 2.0 ml tubes and with adjustable acceleration of up to 14 000 x *g*.
- ✓ Pipette filter tips, for volumes between 1µl and 1 000 µl.
- ✓ Variable volume micropipettes, capable of taking volumes between 1µl and 1 000 µl.
- ✓ Water bath, or other suitable apparatus capable of being maintained at 37°C ±1°C.
- ✓ Water bath, or other suitable apparatus capable of being maintained at 65°C ±1°C.

REAGENTS

- ✓ Anaerobic gas (N_2 85% - H_2 10% - CO_2 5%) or anaerobiosis generation kit.
- ✓ Egg Yolk Agar plates (EYA).
- ✓ Fortified Cooked Meat Medium tubes (FCMM).
- ✓ Nuclease free sterile water.
- ✓ Phosphate-buffered saline (PBS) – pH 7.4.
- ✓ Trypticase-Peptone-Glucose-Yeast extract broth tubes (TPGY).

REAGENT FOR DNA EXTRACTION USING MASTERPURE GRAM POSITIVE DNA PURIFICATION KIT

- ✓ 70% ethanol solution.
- ✓ Isopropanol.
- ✓ MasterPure gram positive DNA purification kit (Epicentre – cat. MGP04020 – MGP04100).

REAGENT FOR DNA EXTRACTION USING BLOOD AND TISSUE KIT

- ✓ Blood and Tissue kit (Qiagen – cat. 69504 – 69506).
- ✓ Ethanol (96-100%).
- ✓ Lysozyme 20 mg/ml.
- ✓ $Na_2EDTA.H_2O$ 2mM.
- ✓ Tris-HCl 20 mM pH 8.0.
- ✓ Triton 100X 1.2%.

REAGENT PREPARATION

<u>TPGY</u>

- Tryptone 50 g
- Peptone 5 g

- Yeast extract 20 g
- D-glucose 4 g
- L-cysteine 0.01 g
- H_2O to 1 000 ml

Dissolve the components in the water by boiling.

Sterilize for 15 min at 121°C. Adjust the pH to 7.0 ±0.2 at 25°C.

Dispense the medium in sterile tubes (9 ml/tube).

Store at refrigerated temperature at 5°C ± 3°C for up to 4 weeks.

To remove oxygen, incubate the tubes in anaerobic conditions for two days or boil them for 10 minutes before use.

FCMM

- Cooked meat medium 1.25 g
- Soluble starch 0.01 g
- Yeast extract 0.1 g
- Calcium carbonate 0.05 g
- Ammonium sulphate 0.1 g
- Glucose 0.08 g
- L-cysteine 0.01 g

Dissolve the components (excluding cooked meat medium) in 10 ml of water.

Sterilize for 15 min at 121°C. Adjust pH to 7.6 ±0.2 at 25°C.

Store at refrigerated temperature at 5°C ± 3°C for up to 4 weeks.

To remove oxygen, incubate the tubes in anaerobic conditions for two days or boil them for 10 minutes before use.

EYA

- Tryptone 5 g
- Peptone 20 g
- Yeast extract 5 g
- NaCl 5 g
- Bacteriological agar 20 g
- H_2O to 1 000 ml

Dissolve the components in water by boiling.

Sterilize for 15 min at 121°C. Adjust the pH to 7.0 ±0.2 at 25°C. Cool the media at 45-50 °C.

Add 80 ml of Egg yolk emulsion.

Distribute in Petri dishes (15-18 ml/dish).

Store at refrigerated temperature at 5°C ± 3°C for 4 weeks.

To remove oxygen, incubate in anaerobic conditions the plates for two days before use.

Phosphate-buffered saline (PBS)

- Na_2HPO_4 1.44 g
- KH_2PO_4 0.24 g
- NaCl 8.00 g
- KCl 0.20 g

Dissolve the reagents listed above in 800 ml of reagent grade distilled H_2O.

Adjust the pH to 7.4 ±0.1 with HCl, and then add H_2O to 1 liter.

Sterilize by autoclaving for 15 min at 121°C.

Store at room temperature.

Lysis Buffer (required for DNA extraction using Blood and Tissue kit)

Preparation of stock solutions

- **$Na_2EDTA.H_2O$ 0.5M pH8.0** – dissolve 186.1 g of $Na_2EDTA.H_2O$ in 800 ml of nuclease free distilled water; adjust the pH using ~ 50 ml of NaOH 10N; adjust the volume using nuclease free water up to 1000 ml; by autoclaving for 15 min at 121°C.
- **Tris-HCl 1M pH 8.0** – dissolve 121.1 g of Tris-base in 800 ml of nuclease free distilled water; adjust the pH using ~ 80 ml of HCl 6N; adjust the volume using nuclease free water up to 1000 ml; sterilize by autoclaving for 15 min at 121°C.

Preparation of Lysis Buffer with Lysozyme

- ✓ Add to 48.2 ml of nuclease free distilled water 1.0 ml of Tris-HCl stock solution, 0.2 ml of $Na_2EDTA.H_2O$ stock solution and 0.6 ml of Triton X100.
- ✓ Sterilize by autoclaving for 15 min at 121°C.
- ✓ Add 1 g of lysozyme.

Lysis buffer with lysozyme should be used the same day of preparation.

PROCEDURE

DNA EXTRACTION USING MASTERPURE GRAM POSITIVE DNA PURIFICATION KIT

- ✓ Pick up a single colony, growth on EYA, using a sterile inoculating loop.

- ✓ Inoculate the single colony in a tube of enrichment culture medium (already incubated in anaerobic conditions for two days).
- ✓ Incubate the tube in anaerobic conditions at 37°C overnight.
- ✓ Chill the culture on ice.
- ✓ Centrifuge 9 ml of the overnight culture at 3 500 x *g* for 15 minutes.
- ✓ Discard the supernatant.
- ✓ Resuspend the cell pellet in 1.5 ml of cold PBS and transfer to a micro-centrifuge tube of 2.0 ml.
- ✓ Centrifuge the cell suspension in a micro-centrifuge at 12 000 x *g* for 5 minutes at 4°C.
- ✓ Discard the supernatant.
- ✓ Resuspend the cell pellet in 1.5 ml of cold PBS.
- ✓ Centrifuge the cells in a micro-centrifuge at 12 000 x *g* for 5 minutes at 4°C.
- ✓ Discard the supernatant.
- ✓ Add 300 µl of TE buffer (included in the kit) and vortex to resuspend cell pellet.
- ✓ Add 2 µl of Ready-Lyse Lysozyme (included in the kit) to the cell suspension.
- ✓ Incubate in water bath at 37°C for 60 minutes.
- ✓ Dilute 2 µl of Proteinase K (50 µg/ µl) into 300 µl of Gram-Positive Lysis Solution.
- ✓ Add 300 µl of the Proteinase K/Gram Positive Lysis Solution to the sample and mix thoroughly.
- ✓ Incubate in water bath at 65°C for 15 minutes, vortexing briefly every 5 minutes.
- ✓ Cool the sample to 37°C.
- ✓ Place samples on ice for 3-5 minutes and then proceed with DNA precipitation.
- ✓ Add 350 µl of MPC Protein Precipitation Reagent to 600 µl of lysed sample and vortex mix vigorously for 10 seconds.
- ✓ Pellet the debris by centrifugation at 4°C for 10 minutes at 14 000 x *g* in a micro-centrifuge.
- ✓ Transfer the supernatant to a clean 2.0 ml micro-centrifuge tube and discard the pellet.
- ✓ Add 2 µl of RNase A (5 µg/ µl) to each sample and mix thoroughly.
- ✓ Incubate at 37°C for 30 minutes.
- ✓ Add 1 ml of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
- ✓ Pellet the DNA by centrifugation at 4°C for 10 minutes at 14 000 x *g* in a micro-centrifuge.
- ✓ Use a pipet tip to remove the isopropanol without dislodging the DNA pellet.
- ✓ Rinse two times the pellet with cool 70% ethanol (stored at -20°C). Centrifuge briefly if the pellet is dislodged.
- ✓ Remove carefully the ethanol from the pellet.
- ✓ Resuspend the DNA in 100 µl of nuclease free sterile water.
- ✓ Store at -70°C the DNA until the use.
- ✓ For shipment, maintain the DNA in frozen condition using dry ice.

DNA EXTRACTION USING BLOOD AND TISSUE KIT

- ✓ Pick up a single colony, growth on EYA, using a sterile inoculating loop.
- ✓ Inoculate the single colony in a tube of enrichment culture medium (already incubated in anaerobic conditions for two days).

- ✓ Incubate the tube in anaerobic conditions at 37°C overnight.
- ✓ Chill the culture on ice.
- ✓ Centrifuge 9 ml of the overnight culture at 5 000 x *g* for 10 minutes.
- ✓ Discard the supernatant.
- ✓ Resuspend bacterial pellet in 180 µl Lysis buffer with Lysozyme.
- ✓ Incubate at 37°C for 60 minutes.
- ✓ Add 180 µl Buffer AL without ethanol (provided in the kit) and add 25 µl proteinase K. (Do not add proteinase k directly to Buffer AL).
- ✓ Mix by vortexing.
- ✓ Incubate at 56°C for 30 minutes.
- ✓ Add 200 µl ethanol (96-100%) to the sample, and mix thoroughly by vortexing (it is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution – A white precipitate may form on addition of ethanol; it is essential to apply all of the precipitate to the DNeasy Mini spin column).
- ✓ Pipet the mixture (including any precipitate) into the DNeasy Mini spin column placed in a 2.0 ml collection tube (provided in the kit).
- ✓ Centrifuge at 6 000 x *g* (8 000 rpm) for 1 minute.
- ✓ Discard flow-through and collection tube
- ✓ Place the DNeasy Mini spin column in a new 2.0 ml collection tube (provided in the kit).
- ✓ Add 500 µl Buffer AW1.
- ✓ Centrifuge at 6 000 x *g* (8 000 rpm) for 1 minute.
- ✓ Discard flow-through and collection tube.
- ✓ Place the DNeasy Mini spin column in a new 2.0 ml collection tube (provided in the kit).
- ✓ Add 500 µl Buffer AW2.
- ✓ Centrifuge at 20 000 x *g* (14 000 rpm) for 3 minutes to dry the membrane.
- ✓ Discard flow-through.
- ✓ Centrifuge again at 20 000 x *g* (14 000 rpm) for 1 minute.
- ✓ Discard flow-through and collection tube.

It is very important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

- ✓ Place the DNeasy Mini spin column in a clean 1.5 ml or 2.0 ml microcentrifuge tube (not provided in the kit) and pipet 200 µl Buffer EB (or 10 mM Tris or nuclease free sterile water) directly into the DNeasy membrane.
- ✓ Incubate at room temperature for 1 minute.
- ✓ Centrifuge at 6 000 x *g* (8 000 rpm) for 1 minute to elute.

Elution with 100 µl (instead 200 µl) increases the final DNA concentration in the elute, but also decrease the overall DNA yield.

- ✓ Store at -70°C the DNA until the use.
- ✓ For shipment, maintain the DNA in frozen condition using dry ice.

22 Appendix 7 : Data reporting forms

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Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation

Form for data collection and reporting

FUR ANIMALS

Version	Written by	Verified by	Approved by
0	Fabrizio Anniballi Bruna Auricchio Stefano Bilei Paola De Santis	All partners	Caroline Le Marechal

anihwa ANIBOTNET Project
Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation

Please return completed questionnaire to:

Dr. ....., Institute.....

address.....

e-mail: ..... Fax: .....

For information please contact: Dr. .....

e-mail: ..... Phone: ..... Fax: .....

DETAILS OF THE COMPILER

First name ..... Surname .....

Institute ..... Address .....

e-mail: ..... Phone: ..... Fax: .....

Date of compilation .....

GENERAL DETAILS

Year ..... Month ..... Country .....

Region/Province ..... Town .....

.....

☐ Suspected case ☐ Suspected outbreak

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☐Minks ☐Ferrets ☐Foxes ☐Other (specify) .....

Animal species involved .....

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Total number of animals in the farm or in the flock .....

Number of affected animals .....

Number of died animals .....

CLINICAL DETAILS

Neurological signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Gastrointestinal signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Systemic signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Clinical History: briefly describe history and general symptomatology progression:

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Laboratory Results:

Was blood analysis done? ☐Yes ☐No

Please describe haematological and bio-chemical findings

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Was a necropsy done? ☐Yes ☐No

Please describe the most significant findings

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HOUSING

☐Non-cage system	Pens	☐fully covered by litter
		☐partially covered by litter
		☐perforated floor
		☐combined
	Furnishings	☐nest
		☐other (specify).....
		☐other (specify).....
		☐other (specify).....
☐Cage system	Furnishings	☐nest
		☐wood branches
		☐plastic tubes
		☐straw
		☐other (specify).....
		☐other (specify).....
		☐other (specify).....

Material used for manure's drainage below of the cages

☐straw

☐wood chips

☐other (specify).....

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MICROCLIMATE AND ENVIRONMENTAL CONDITIONS

Ventilation ☐ natural ventilation

☐ mixed ventilation No. air movements/h: .....

☐ forced ventilation: No. air movements/h: .....

Air temperature: .....

Relative humidity: .....

Water temperature: .....

Overall air quality: .....

Dust level: .....

FEEDING

Feeding modalities ☐ manual distribution

☐ automatic distribution

Drinkers ☐ drinking bath

☐ nipple drinker

Diet ☐ dry diet

☐ wet diet

diet composition .....

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OTHER FEEDING DETAILS

Please describe the most significant fed detail and change in the 7 days before the symptomatology onset

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LABORATORY ANALYSIS FOR CONFIRMATION OF BOTULISM SUSPICION

Clinical specimens:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Serum			
Rectal faeces			
Faeces			
Intestinal content			
Intestine			
Liver			
Spleen			
Heart			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Feed samples:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Dry feed			
Wet feed			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Environmental samples:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Drinker (swab)			
Drinker (water)			
Water system (swab)			
Water system (water)			
Air system (swab)			
Farm wall (swab)			
Cage (swab)			
Feeding dispenser (swab)			
Feeding dispenser (feed)			
Feeding wagon (swab)			
Feeding wagon (feed)			
Feeding area (floor swab)			
Nest (swab)			
Pen (soil)			
Surrounding area			
Small animal carcasses			
Decomposing organic matter			
Maggots			
Drainage manure material below cages			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Laboratory details:

Laboratory .....

Address .....

Laboratory director .....

e-mail: ..... Phone: ..... Fax: .....

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Form for data collection and reporting

POULTRY

Version	Written by	Verified by	Approved by
0	Fabrizio Anniballi Bruna Auricchio Stefano Bilei Paola De Santis	All partners	Caroline Le Marechal

Please return completed questionnaire to:

Dr. ..... Institute .....

address .....

e-mail: ..... Fax: .....

For information please contact: Dr. .....

e-mail: ..... Phone: ..... Fax: .....

DETAILS OF THE COMPILER

First name ..... Surname .....

Institute ..... Address .....

e-mail: ..... Phone: ..... Fax: .....

Date of compilation .....

GENERAL DETAILS

Year ..... Month ..... Country .....

Region/Province ..... Town .....

.....

☐ Suspected case ☐ Suspected outbreak

☐ Broiler ☐ Laying hens ☐ Turkeys ☐ Pheasants ☐ Other (specify) .....

Animal species involved .....

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Total number of animals in the farm or in the flock .....

Number of affected animals .....

Number of died animals .....

CLINICAL DETAILS

Neurological signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Gastrointestinal signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Systemic signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Clinical History: briefly describe history and general symptomatology progression:

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Laboratory Results:

Was blood analysis done? ☐ Yes ☐ No

Please describe haematological and bio-chemical findings

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Was a necropsy done? ☐ Yes ☐ No

Please describe the most significant findings

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HOUSING

☐ Cage system
- ☐ unenriched cage system (conventional laying cages)
- ☐ enriched cage system (furnished cages)

☐ Non-cage system
- ☐ indoor single level
  - ☐ fully covered by litter
  - ☐ partially covered by litter
  - ☐ perforated floor
  - ☐ combined
- ☐ indoor multilevel
  - ☐ fully covered by litter
  - ☐ partially covered by litter
  - ☐ perforated floor
  - ☐ combined
- ☐ outdoor multilevel
  - ☐ covered verandas
  - ☐ free-range
- ☐ fully litter floor system

SHED - LITTER

Deep litter
- ☐ No
- ☐ Yes amount of consumed litter per day .....
  frequency of litter distribution .....
  frequency of litter removal .....

☐ Floor type
- ☐ concrete soil
- ☐ slatted floor
- ☐ grooved floor

Covering material
- ☐ wood chips
- ☐ straw
- ☐ sand
- ☐ compost
- ☐ other (specify) .....

MICROCLIMATE AND ENVIRONMENTAL CONDITIONS

Ventilation
- ☐ natural ventilation
- ☐ mixed ventilation No. air movements/h: .....
- ☐ forced ventilation: No. air movements/h: .....

Air temperature: .....
Relative humidity: .....
Dust level: .....
Overall air quality: .....
Water temperature: .....

FEEDING

Feeders
- ☐ inside the area accessible to the birds
- ☐ outside the area accessible to the birds
- ☐ individual electronic concentrate dispenser

Drinkers
- ☐ open water system
- ☐ closed water system
  - ☐ cup drinker
  - ☐ nipple drinker

Diet
- ☐ dry diet
- ☐ birdseeds
- ☐ grass
- ☐ other (specify) .....

OTHER FEEDING DETAILS

Please describe the most significant feeding detail and change in the 7 days before the symptomatology onset

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ENVIRONMENTAL DETAIL

☐ Wet zones or presence of wet zones near the area in which the case/outbreak occurs

☐ Presence of died animals in the area in which the case/outbreak occurs

If yes, died animals were promptly removed? ☐ Yes ☐ No

☐ Presence of maggots in the area in which the case/outbreak occurs

If yes, died animals were promptly removed? ☐ Yes ☐ No

Other significant environmental features (e.g. type of soil; pH of soil; water temperature; etc.)

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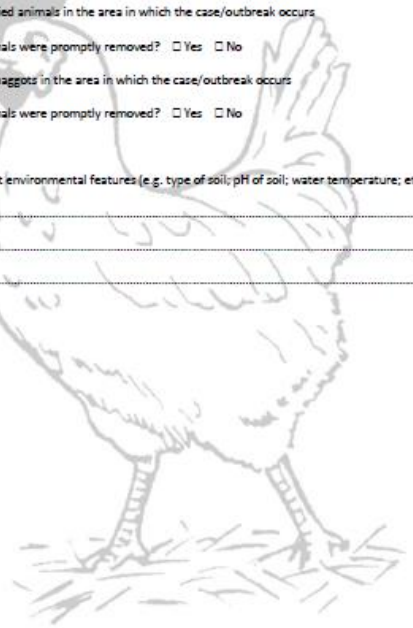

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Form for data collection and reporting

CATTLE

Version	Written by	Verified by	Approved by
0	Fabrizio Anniballi Bruna Auricchio Stefano Bilei Paola De Santis	All partners	Caroline Le Marechal

Please return completed questionnaire to:

Dr. ..... Institute.....

address.....

e-mail: ..... Fax: .....

For information please contact: Dr. .....

e-mail: ..... Phone: ..... Fax: .....

DETAILS OF THE COMPILER

First name ..... Surname .....

Institute ..... Address .....

e-mail: ..... Phone: ..... Fax: .....

Date of compilation .....

GENERAL DETAILS

Year ..... Month ..... Country .....

Region/Province ..... Town .....

.....

☐ Suspected case ☐ Suspected outbreak

☐ Cows ☐ Beef ☐ Calves ☐ Other (specify) .....

Animal species involved .....

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Total number of animals in the farm or in the flock .....

Number of affected animals .....

Number of died animals .....

CLINICAL DETAILS

Neurological signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Gastrointestinal signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Systemic signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Clinical History: briefly describe history and general symptomatology progression:

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Laboratory Results:

Was blood analysis done? ☐ Yes ☐ No

Please describe haematological and bio-chemical findings

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Was a necropsy of died animals done? ☐ Yes ☐ No

Please describe the most significant findings

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MICROCLIMATE AND ENVIRONMENTAL CONDITIONS

Ventilation
☐ natural ventilation
☐ mixed ventilation No. air movements/h: .....
☐ forced ventilation: No. air movements/h: .....

Air temperature: .....

Relative humidity: .....

Dust level: .....

Overall air quality: .....

Water temperature: .....

FEEDING

Feeding
☐ self-feeding
☐ individual electronic feeding
☐ individual electronic concentrate dispenser

Drinkers
☐ open water system
☐ closed water system ☐ cup drinker ☐ nipple drinker

Diet
☐ wet fermented diet
☐ silage
☐ dry diet
☐ hay
☐ legume-based forage
☐ high dietary protein intake

OTHER FEEDING DETAIL

Please describe the most significant feeding detail and change in the 7 days before the symptomatology onset

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ENVIRONMENTAL DETAIL

☐ Use of polline as field fertilizer of pasture or forage areas ☐ Yes ☐ No

☐ Wet zones or presence of wet zones near the area in which the case/outbreak occurs

☐ Presence of died animals in the area in which the case/outbreak occurs

If yes, were died animals promptly removed? ☐ Yes ☐ No

☐ Presence of maggots in the area in which the case/outbreak occurs

If yes, died animals were promptly removed? ☐ Yes ☐ No

Other significant environmental features (e.g. type of soil; pH of soil; water temperature; etc.)

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LABORATORY ANALYSIS FOR CONFIRMATION OF BOTULISM SUSPICION

Clinical/biological specimens:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Serum			
Rectal faeces			
Faeces			
Intestinal content			
Intestine			
Stomach (specify) content			
Liver			
Spleen			
Udder milk			
Tank milk			
Udder tissue			
Heart			
Diaphragm			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Feed samples:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Ensilage			
Fodder			
Hay			
Birdseed			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Environmental samples:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Drinker (swab)			
Drinker (water)			
Water system (swab)			
Water system (water)			
Air system (swab)			
Farm wall (swab)			
Feeding dispenser (swab)			
Feeding dispenser (feed)			
Feeding wagon (swab)			
Feeding wagon (feed)			
Feeding area (floor swab)			
Pasture area			
Open feedlot			
Bedding area			
Lying down area			
Milking parlour (swab)			
Exercise area (swab)			
Mud			
Maggots			
Decomposing organic matter			
Small animal carcasses			
Surrounding area			
Other (specify)			
Other (specify)			

Laboratory details:

Laboratory .....

Address .....

Laboratory director .....

e-mail: .....

Phone: ..... Cell..... Fax: .....

Form for data collection and reporting

WILD BIRDS
OTHER ANIMALS

Version	Written by	Verified by	Approved by
0	Fabrizio Anniballi Bruna Auricchio Stefano Bilei Paola De Santis	All partners	Caroline Le Marechal

Please return completed questionnaire to:

Dr. ....., Institute.....

address.....

e-mail: ..... Fax: .....

For information please contact: Dr. .....

e-mail: ..... Phone: ..... Fax: .....

DETAILS OF THE COMPILER

First name ..... Surname .....

Institute ..... Address .....

e-mail: ..... Phone: ..... Fax: .....

Date of compilation .....

GENERAL DETAILS

Year ..... Month ..... Country .....

Region/Province ..... Town .....

.....

☐ Suspected case ☐ Suspected outbreak

☐ Farmed animals ☐ Wild animals ☐ Pet animals

Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation

Animal species involved .....

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Total number of animals in the farm or in the flock .....

Number of affected animals .....

Number of died animals .....

CLINICAL DETAILS

Neurological signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Gastrointestinal signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Systemic signs:

Clinical sign	Onset date of clinical sign	N. of affected animals

Clinical History: briefly describe history and general symptomatology progression:

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Laboratory Results:

Was blood analysis done? ☐ Yes ☐ No

Please describe haematological and bio-chemical findings

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Was a necropsy done? ☐ Yes ☐ No

Please describe the most significant findings

.....

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.....

.....

ENVIRONMENTAL DETAIL

☐ Wet zones or presence of wet zones near the area in which the case/outbreak occurs

☐ Presence of died animals in the area in which the case/outbreak occurs

If yes, died animals were promptly removed? ☐ Yes ☐ No

☐ Presence of maggots in the area in which the case/outbreak occurs

If yes, died animals were promptly removed? ☐ Yes ☐ No

Temperature .....

Humidity .....

Other significant environmental features (e.g. type of soil; pH of soil; water temperature; etc.)

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Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation

LABORATORY ANALYSIS FOR CONFIRMATION OF BOTULISM SUSPICION

Clinical specimens:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Serum			
Rectal faeces			
Faeces			
Intestinal content			
Intestine			
Stomach (specify) content			
Liver			
Spleen			
Udder milk			
Hearth			
Diaphragm			
Udder tissue			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Feed samples:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Ensilage			
Fodder			
Hay			
Birdseeds			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Environmental samples:

Type of sample	N. of tested samples	N. of positive samples (BoNT - type)	N. of positive samples (Organism)
Water			
Mud			
Soil			
Maggots			
Decomposing organic matter			
Small animal carcasses			
Benthic invertebrate			
Worms			
Drinking area			
Water system			
Feeding area			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			
Other (specify)			

Laboratory details:

Laboratory .....

Address .....

Laboratory director .....

e-mail: ..... Phone: ..... Fax: .....

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Form for cattle suspicion used by the French NRL

Name :
Name of the person in charge of sample collection : Sampling Date :
Farm address : Veterinarian:

Clinical signs :

☐ Apathy ☐ Dysphagia ☐ Constipation
☐ Hypersalivation ☐ Rear end paralysis ☐ Diarrhoea
☐ Weakness ☐ Recumbency ☐ Tongue paralysis
☐ Anorexia ☐ Tail paralysis ☐ Tremors
☐ Paralysis of the jaw muscles ☐ Respiratory failure

Animal death : Date : Hour :

☐ Natural ☐ Euthanasia

Number of animals with clinical signs :……
Number of dead animals :……..
Other suspected diseases :……
High or low suspicion ?

Context :

☐ Previous botulism outbreak on the farm ☐ vaccination against botulism
☐ Poultry on the farm
☐ Poultry next to the farm
☐ Poultry manure recently spread next to the farm
☐ Wild birds are present on the farm
☐ Detection of a carcass in one of the ration components

Composition de la ration : ☐ Mixing wagon is used

☐ Grass silage or wrapped grass ☐ Pasture
☐ Corn silage
☐ Cereals Specify :…… ☐ Use of a mill (flour)
☐ Concentrate ☐ Use of a worm screw
☐ Hay
☐ Other : ……..

Does one of the components present an unusual aspect (smell, colour, texture…)
……