

Type C bovine botulism outbreak due to carcass contaminated non-acidified silage

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Running head: Type C bovine botulism outbreak

Keywords: *Clostridium botulinum*, botulism, cattle, silage, AFLP, outbreak, animal carcass

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SUMMARY

The first reported bovine botulism outbreak in Finland is described. Nine of 90 cattle on a dairy farm died after being fed non-acidified silage contaminated by animal carcasses. Type C botulinum neurotoxin gene was detected in one heifer by polymerase chain reaction (PCR) and the neurotoxin was detected by the mouse bioassay. *Clostridium botulinum* type C was isolated from liver samples. The isolated strain was identified with amplified fragment length polymorphism analysis (AFLP) as Group III *C. botulinum*. To our knowledge, this is the first time that a type C bovine botulism outbreak has been diagnosed by PCR and confirmed by subsequent isolation and AFLP identification of the disease strain. The importance of the acidification process in silage production to inhibit *C. botulinum* toxin production in silage and thus to prevent further botulism outbreaks is emphasized. Nevertheless, preformed toxin in i.e. carcass is not destroyed by acid.

INTRODUCTION

Botulism is a severe neurological disease affecting cholinergic nerve-endings by blocking acetylcholine transport via the synaptic cleft and ultimately causing muscle paralysis and frequently death. Botulism is caused by the botulinum neurotoxin (BoNT), which is produced by the facultative anaerobic spore-forming bacterium *Clostridium botulinum*. BoNT, the most potent toxin known, is produced under anaerobic conditions at appropriate high pH: growth and toxin production may occur in foods, feeds, deep wounds or in a neonatal or disturbed adult intestinal tract. BoNTs are divided into seven serologically distinct types, A to G, that cause disease in humans, non-human mammals, birds and fish. The species *C. botulinum* organisms is divided into four physiologically distinct Groups I-IV. Group I strains produce neurotoxin types A, B, and F; Group II strains produce toxins B, E, and F; Group III strains produce neurotoxin types C and D; and Group IV strains produce G toxin. Types A, B, C and D toxins have been reported to cause disease in cattle [1-2], while human botulism is caused mostly by types A, B, E and F [3]. Botulism intoxication, caused by a preformed and ingested toxin, is known as classical botulism – the most typical form affecting animals and humans.

C. botulinum spores are ubiquitous in the environment and a high prevalence has been reported in sediments and in the intestinal tract of animals in Nordic countries [4-6]. Therefore grass used as cattle feed or animal carcasses in feed may be contaminated by *C. botulinum*, and the organism or its neurotoxin is often found in fodder [7-10]. *C. botulinum* type B is known to grow and produce neurotoxin in grass feed [11], whereas *C. botulinum* types C and D botulism cases are typically related to carcass contaminated feed. However, evidence on *C. botulinum* types C and D being able to use plant material as a substrate for growth has been published [12]. In general, cattle are at risk when feeding on anaerobically stored silage with a pH above 5.5, or on acidified silage containing animal carcasses which may support growth and toxin production. An estimated 1 g of botulinum neurotoxin could potentially kill 400 000 adult cows [10].

In many countries, botulism is a major problem on cattle farms, and in recent years many outbreaks have been described with some cases involving the deaths of hundreds of animals, resulting in enormous economic losses [1,8-10,13,14]. In England and Wales, with a cow population of 1 300 000, a total of 55 bovine botulism outbreaks occurred in 2003-2005 [14]. In certain countries, the risk is considered to be so large that dairy cows are vaccinated yearly against the disease. In Israel, the dairy cow population is 110 000 and a comprehensive vaccination programme against botulism types C and D for dairy cows was already in place by 1977 [15]. The rising number of botulism outbreaks in cows is disturbing and practices to prevent further cases are being evaluated [14].

The diagnosis of bovine botulism relies mostly on typical clinical signs (flaccid tongue, decreased muscle tone, dilated pupils), but laboratory confirmation is required to rule out differential diagnoses [1]. While laboratory methodology in bovine botulism diagnostics includes ELISA and other immunological tests [16], toxin detection with the mouse bioassay remains the standard [1]. However, use of the mouse bioassay is ethically questionable, and the development of new sensitive laboratory methods has been recommended [14]. Recently, PCR methods have been introduced as sensitive and rapid diagnostic tests for investigating bovine botulism [17-19]. Fach *et al.* [17] established a double PCR method for testing naturally contaminated clinical samples as a reliable alternative to the mouse bioassay. This study describes the first type C bovine botulism outbreak diagnosed using PCR and confirmed

with subsequent isolation and identification of the disease strains by AFLP. This is the first reported bovine botulism outbreak in Finland which has a cow population of 320 000.

MATERIALS AND METHODS

Case history

On 27 July 2006, sudden signs of anorexia and ataxia in a heifer were observed by a farmer on a dairy farm with 90 cattle in western Finland. A veterinary officer was called to the farm, but the heifer died before any veterinary care could be given. On day 2, two other animals, cow no. 1 and heifer no. 2, showed similar signs and a local veterinary officer was called to the farm. These animals were observed to be anorexic, weak, dysphagic and paretic and were given veterinary care following a diagnosis of grass tetany. Over a period of 3 weeks, a total of six cows and three heifers with similar signs died or were euthanized despite treatment. Most of the cattle were treated with intravenous calcium, magnesium, ketoprofen, and vitamin B infusion, but no significant improvement was observed during the treatment. The most evident and commonly observed clinical signs were anorexia, ataxia, dysphagia, weakness, paresis and sternal recumbency (Table 1). Since the pH of the ruminal contents of two cows was above 8 and the number of affected cattle increased, feed poisoning and indigestion were the main suspected differential diagnoses. No deficiencies in feed quality were found. On day 11, the farmer was advised to give peroral fluid therapy to aphagic cow no. 3. On day 12, cow no. 3 died and the local veterinary officer conducted a necropsy investigation. On days 12 and 13, the carcasses or organ samples of heifer no. 3 and cows no. 3 and 4 were sent for necropsy to the Department of Basic Veterinary Sciences (DBVS), and botulism specialists at the Department of Food and Environmental Hygiene (DFEH) were consulted about the outbreak. On day 16, clinical investigation revealed the tongues of cows no. 5 and 6 to be flaccid, i.e. they could easily be pulled out of the animals' mouths and botulism was suspected as the main diagnosis.

Investigations on the farm

Besides the local veterinary officer visiting the farm several times during the outbreak, an epidemiological survey was conducted on the farm to investigate conditions, the development of the outbreak, feeding routines, feed manufacturing, cowshed layout and other epidemiological matters.

Pathology and samples

The carcasses of two animals, heifer no. 3 (weight 332 kg) and a 4-year-old Ayshire cow (no. 4- weight 420 kg) that was pregnant with a male foetus, were submitted to the DBVS for necropsy. The heifer was received first and the cow one day later. Routine histopathological samples were collected, fixed and processed. Samples of the gastrointestinal tract and liver were also submitted for *C. botulinum* examination at the DFEH. In addition, samples of silage and the gastrointestinal tract of cow no. 3, which was necropsied on the farm, were sent by the local veterinary officer to the DFEH for *C. botulinum* examination.

Detection of botulinum neurotoxin gene by PCR

In order to compare the different pretreatments for PCR, gastrointestinal and liver samples from the three cattle and a silage sample were inoculated into a tryptose-peptone-glucose-yeast (TPGY) broth, on plates of egg-yolk agar (EYA) and on blood agar. A total of 1 g of each sample material was transferred to 10-ml TPGY broth tubes. EYA and blood agar plates

were cultured directly from sample material with and without ethanol treatment to select the spores [20]. Tubes and plates were incubated under anaerobic conditions at 30°C and 37°C for 3 days, after which 1 ml of the anaerobic TPGY culture or colonies from the plates were transferred to 10 ml of fresh TPGY medium for overnight incubation at the respective temperatures. After incubation, 1 ml of each overnight culture was transferred to 1.5 ml tubes. The tubes were centrifuged for 3 min at 13 000 x g, and the supernatant was replaced with 1 ml of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA). Tubes were incubated for 1 h at 37°C, centrifuged for 3 min at 13 000 x g and the cells were suspended in 1 ml of distilled water. Suspensions were heated at 95°C for 10 min to release the DNA and then centrifuged for 5 min at 13 000 x g. A volume of 1-3 µl of each heated supernatant was used as a template in PCR examinations.

C. botulinum type A, B, E and F neurotoxin genes (*botA*, *botB*, *botE* and *botF*) were investigated by the multiplex PCR method described by Lindström *et al.* [21], *C. botulinum* type C neurotoxin gene (*botC*) was investigated by nested PCR as described by Williamson *et al.* [22], and *C. botulinum* type D neurotoxin gene (*botD*) was investigated by the PCR method as described by Szabo *et al.* [23].

Isolation of *C. botulinum* strains

A volume of 0.1 ml of each PCR-positive overnight culture was plated onto EYA and blood agar plates, and incubated anaerobically at 30 and 37 °C for 3 days. Carriage of *botC* by lipase-positive colonies was confirmed by PCR [22].

DNA extraction and AFLP

PCR-positive *C. botulinum* isolates were subjected to DNA extraction [24,25] and AFLP analysis, as described previously by Keto-Timonen *et al.* [25]. Lysozyme, mutanolysin and RNase were supplied by Sigma (St. Louis, MO), proteinase by Finnzymes (Espoo, Finland), restriction enzymes and ligase by New England Biolabs (Beverly, MA) adapters and primers by Oligomer (Helsinki, Finland). The AFLP fingerprints were compared with the clostridial AFLP library at the DFEH. A cut-off value of 95% was used to indicate strain identity [25].

Mouse bioassay

PCR-positive samples from dead cattle and silage were tested for the presence of botulinum neurotoxin by the mouse bioassay method approved by the Nordic Committee of Food Analysis [26]. In brief, sample eluate was injected into mice intraperitoneally. Mice were observed for 4 days for the presence of typical clinical signs (muscle weakness, respiratory problems, wasp waist), and euthanized immediately after observation of such signs. To confirm the botulinum neurotoxin to be of type C, neutralizing type C antibodies were incubated with the toxic sample eluate, and the solution or boiled eluate was injected into mice [27].

pH measurement

The pH of each sample was measured (Microprocessor pH 537; Wissenschaftlich- Technische Werkstätten, Weilheim, Germany) following homogenization with distilled water (1:1 [wt/vol]).

RESULTS

Epidemiological investigations on the farm

Cows on the farm were housed indoors in a cowshed before the outbreak. Silage was offered ad libitum to cattle and one silage bale was consumed in one pen row each day. One day before the first clinical signs were observed, small pieces of rotten animal carcasses were found by the farmer in the silage provided to one feeding passage. The carcasses were likely to have been incorporated into silage while grass harvesting. The carcasses were probably fragmented during processing of silage, which is usually chopped into pieces around 10 cm in length. The carcass species was not identified. The feeding passage was cleaned and the rest of the bale was taken outside to the pastures to decompose. However, the cattle of this pen row had already been exposed to the silage. In three other pen rows, no dairy cows became ill. A total of 16 dairy cows were situated in the same pen row, six of which manifested the clinical signs listed in Table 1 over the next 3 weeks and were ultimately euthanized or died. During the 3-week period three calves and heifers showed similar symptoms and died or were euthanized. The affected cows were located next to each other, and the affected heifers and calves used the same feeding passage from the opposite side. The possibility that more than one bale was contaminated with botulinum toxin cannot be excluded, but only one bale with pieces of a rotten carcass was identified as being fed to animals.

Assuming that all intoxications were due to silage given on 26 July, incubation times varied from 1 to 10 days among heifers and from 1 to 16 days among cows. Five animals died 0-4 days after the first clinical signs manifested, and three animals were euthanized 2-5 days after the onset of clinical signs.

According to the farmer, the season had been exceptionally dry and the grass was harvested dry; hence, the silage was prepared without the usual acidification procedure. The green grass was harvested at night-time, chopped and compressed in bales weighing 400-500 kg and wrapped in plastic sheeting to create anaerobic conditions in the bale. When the new silage batch first arrived at the farm, the farmer noticed small pieces of animal carcass under the plastic sheet of two bales. These bales were not used as feed and were thrown away. In normal feeding routines on the farm, silage was offered ad libitum. Silage bales were unwrapped at the end of a feeding passage and silage was manually distributed over the entire length of the passage for cows and young cattle. Cattle were also offered commercial concentrated feed and dry hay. Concentrated feed was offered as a dry feed, containing approximately 85% of dry matter.

The farm had raised poultry some decades earlier. However, according to the farmer, poultry carcasses or litter had not been buried in the field where the silage was gathered.

Necropsy investigations

There were few significant necropsy findings. Heifer no. 3 and cow no. 4 showed marked hyperaemia in the mucosa of the abomasum, duodenum and jejunum. Petechial haemorrhages were detected in the rumen and omasum. Routine microbiological cultures performed on tissues from both animals were negative for any specific bacteria, showing only mixed flora. In addition to gross changes, mild centrilobular hepatic degeneration was detected in one cow. In nervous tissue, autolytic changes were seen, but there were no significant lesions.

Laboratory analyses

PCR

Upon PCR examination, *botC* was detected in the abomasal content, intestinal wall, intestinal content and liver of heifer no. 3 (Table 2). When PCR pretreatments were compared, the samples incubated on EYA plates or TPGY broth yielded more PCR-positive isolates than the samples incubated on blood agar plates (Table 3). No differences between samples prepared with and without ethanol treatment were detected.

Mouse bioassay

Type C botulinum neurotoxin was detected by the mouse bioassay in the liver, intestinal content and abomasal content of heifer no. 3, but not in the blood sample of cow no. 4 (Table 2).

Isolation

Lipase-positive colonies were isolated from the PCR-positive liver sample. Two isolates (CBO1C-1 and CBO1C-2) were shown to carry the *botC* gene by PCR analysis (Table 2). *C. botulinum* was not isolated from the two other bovines or from the silage samples (Table 2).

AFLP analysis

Based on AFLP analysis, *C. botulinum* isolates CBO1C-1 and CBO1C-2 clustered together with the DFEH Group III *C. botulinum* type C library strains. The similarity between the two bovine isolates and the other *C. botulinum* type C strains varied from 41% to 84%. All *C. botulinum* Group III strains were linked together with a similarity of 35% (Fig. 1). The AFLP profiles of CBO1C-1 and CBO1C-2 isolates had a similarity of 95%, indicating that the strain profiles were identical.

pH

The pH of the silage sample was 5.97, and the pH of different gastrointestinal samples varied from 5.73 to 8.04 (Table 2).

DISCUSSION

To our knowledge, this is the first time that a type C bovine botulism outbreak has been diagnosed using PCR and subsequently confirmed with isolation and identification of the organisms as Group III *C. botulinum* type C by AFLP analysis. The *botC* gene was detected by PCR [22] in the intestinal content, intestinal wall, abomasal content and liver of one dead heifer. The intestinal content, abomasal content, and liver of the dead heifer were also positive for type C botulinum toxin in the mouse bioassay. Based on these findings, PCR is a very useful confirmatory tool for investigating bovine botulism outbreaks.

C. botulinum type C was isolated from the liver samples, but not from the rumen, abomasum or intestines. Isolation of Group III *C. botulinum* is challenging due to its strict anaerobic culture requirements [38]. This is especially the case with gastrointestinal samples, which have a large number of competing microbes. Moreover, the location of the neurotoxin gene on a bacteriophage makes isolation of toxin producing organisms challenging since conditions which cause the phage to be lost are not fully understood.

In AFLP analysis the *C. botulinum* isolates CBO1C-1 and CBO1C-2 were shown to be identical and clustered together with *C. botulinum* Stockholm C library strain at a similarity level of 84%. The use of AFLP has not previously been reported for identifying isolates from bovine botulism outbreaks; however, the present findings show that AFLP is a useful tool for identifying and characterizing disease isolates in outbreak studies.

The samples taken from silage and from the two animals that died were negative for *botC* in the PCR and for toxin in the mouse bioassay. The silage sample was taken from a silage batch harvested at night, but not from the bale containing pieces of animal carcass which was given to animals on 26 July, suggesting that only a limited number of bales were contaminated. As all the affected animals were next to each other in the pen row where the contaminated bale was placed, while animals in other pen rows were not affected, the intoxication was likely due to one or only a few bales. The fact that the bovine samples were negative in the mouse bioassay might be explained by toxin degradation in the 3-4 days between death, sampling and analysis. Allison *et al.* [29] reported the ability of ruminal microbes to rapidly inactivate botulinum type C toxin. In addition, the sensitivity of the mouse bioassay may be too low to detect botulism in many animals [1]. Negative PCR results may be explained by the presence of inhibitory substances in the intestinal contents.

The time period in which the nine cattle were affected was 16 days, leading to the question of whether more than one bale was contaminated. In the literature, the incubation period in cows has typically been reported to be 12-48 h. Ingestion of large amounts of neurotoxin may cause clinical signs within 12-24 h, but lower toxin concentrations may not cause clinical signs for 7-10 days or longer [1]. In a Californian type C botulism outbreak on a dairy farm, deaths continued for as long as 19 days after a contaminated feed was fed [10]. The long incubation times may be explained by biphasic pathogenesis. Neill *et al.* [30] reported a biphasic bovine botulism outbreak in which acute onset of clinical signs occurred within 72-96 h, but some animals became affected 2 weeks after exposure to the contaminated feed. They concluded that acute intoxication had a lethal effect on some cattle, but for some other cattle, intoxication only debilitated them transiently, providing conditions for slow toxin production *in vivo* and thus causing toxicoinfection.

The field necropsy investigation revealed that cow no. 3 had died of aspiration pneumonia. This is not unexpected, as botulism was not suspected at the time that the farmer was advised to give peroral fluids to the cow. In botulism, laryngeal muscles are paralysed causing dysphagia, and fluids are readily taken into the trachea and lungs if the administration technique is not appropriate. No other macroscopic lesions were observed, which is in line with the literature [1]. The paucity of post mortem lesions in the two other bovines is typical of botulism reflecting the pathogenesis of the disease; toxins altering synaptic function do not cause morphological lesions in neurons or organs.

This is the first reported botulism outbreak in cattle in Finland. A reason why outbreaks have not occurred earlier in Finland might be related to the feed manufacturing process. Silage and silage-based fodders are often reported to be a vehicle in botulism outbreaks [1, 8-10]. In Finland, silage is usually acidified, ideally to a pH of 3.8-4.0, and wrapped with plastic. Despite anaerobic conditions in the bale, the low pH inhibits the growth of *C. botulinum* [31]. Acidified grass silage became the main fodder in Finland for cattle after the A-I-V system was introduced in 1933 [32], but in many countries silage is manufactured without the addition of acid, and there is reliance on the fermentation process to decrease the pH to below 4.5. For instance, in the United States, an acidifying process is not recommended for silage [33]. However, a drawback in using non-acidified silage is the occasional failure of the fermentation process, leading to an excessively high pH, with the risk of botulism outbreaks increasing as a result [9]. In the present outbreak, unusually the silage was prepared without acidification and its pH was found to be 5.97, providing *C. botulinum* with favorable conditions to grow and produce neurotoxin. In Finland, the importance of the acidification process has been recognized earlier with fur animal feed; in 2002, type C botulism affected

more than 52 000 fur animals as a result of non-acidified feed components containing *C. botulinum* type C and its neurotoxin [34]. The botulism risk in fur animal practices is usually prevented with chopping and acidifying the feed raw materials such as slaughterhouse by-products. In view of these findings, an acidifying procedure should be used when manufacturing animal feed to ensure the development of sufficiently low pH.

C. botulinum spore contamination in silage is an important issue when assessing the botulism hazard for livestock. *C. botulinum* is ubiquitous in the environment, and soil is often reported to contain spores of *C. botulinum* type B, C or D [35]. Silage contamination by soil during harvesting cannot be avoided, and therefore there is always a risk of contamination with *C. botulinum*. Notermans *et al.* [7] reported in 1981 that *C. botulinum* type B can be cycled from a contaminated pasture to grass silage and cows, thereby causing a botulism hazard. *C. botulinum* spores may also contaminate silage via carcasses of dead animals. Many botulism outbreaks have been reported to be caused by feed contaminated by carcass material [10]. In the present case, pieces of animal carcasses were found in the silage, and two bales had already been thrown away from the same batch because of carcass contamination, instead of being used as feed. *C. botulinum* spores are often detected in the intestinal tract of animals [5,6,36], hence carcass contamination of feed is a clear risk, which should be prevented. The present silage batch was prepared at night, when many nocturnal animals wander on fields of grass and can easily be crushed in the silage during harvesting. The role of poultry in contaminating the silage cannot be excluded since the farm had raised poultry a few decades earlier. Poultry litter, especially that containing carcasses, has been reported to be associated with bovine botulism [37] when cattle have had access to stored litter or when litter has been spread on grazing fields as a fertilizer. Although poultry production had ceased many years earlier on the farm, *C. botulinum* spores may have persisted in grass pastures, contaminating feed, grazing cattle or wild animals. The intestinal tracts of wild animals grazing in contaminated fields have been shown to be easily contaminated with *C. botulinum* [7], and might therefore maintain the endemic population of *C. botulinum* and serve as a reservoir for the spores.

C. botulinum types A and B are known to use plant material, such as grass, as a substrate for growth, and therefore acidification of feed is expected to be important to prevent type A and B botulism outbreaks in cattle. Opposing reports on the ability of *C. botulinum* type C to grow and produce toxin in plant material have been published [11-12]. Nevertheless, *C. botulinum* type C organisms failed to grow and produce toxin in grass material. Hence acidification is suggested to control the botulism risk related to the growth and toxin production by types A and B, but also by types C and D, in feed. However, type C and D botulism outbreaks are typically related to carcass contamination of feed. Therefore acidification does not necessarily eliminate the risk of toxin formation in a carcass, unless it is chopped into small pieces, exposing *C. botulinum* cells or spores present in the carcass gastrointestinal tract to the acid. Moreover, decomposing carcasses may contain large amounts of preformed neurotoxin which may be further spread to the feed upon processing. It is important to bear in mind that an acidic pH does not destroy the neurotoxin [38].

In conclusion, PCR provided a useful confirmatory tool for laboratory diagnosis of bovine botulism. AFLP was shown to be a valuable method in identifying isolates from a botulism outbreak as *C. botulinum*. The importance of the acidification process in silage production to prevent *C. botulinum* growth and toxin production in silage is emphasized. To enhance the inhibitory effect of acid, grass, and contaminating carcasses in it, should be chopped into small pieces upon harvest for efficient exposure of *C. botulinum* to acid.

ACKNOWLEDGEMENTS

The authors thank Mari Nevas, DVM, PhD, for scientific discussions and Hanna Korpunen for excellent laboratory assistance.

DECLARATION OF INTEREST

None.

References

1. **Whitlock RH, Williamson JL.** Botulism toxicoses of cattle. *Proceedings of the 32nd Annual Conference of the American Association of Bovine Practitioners* 1999; **32**: 45-53.
2. **Schocken-Iturrino RP, et al.** First case of type A botulism in zebu. *The Veterinary Record* 1990; **3**: 217-218.
3. **Allen SD, Emery CL, Lyerly DM.** *Clostridium*. In Murray PR, et al. eds. *Manual of Clinical Microbiology*. 8th ed. Washington DC: ASM Press; 2003: pp. 835-856.
4. **Hielm S, et al.** A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and baltic sea sediment samples. *Journal of Applied Microbiology* 1998; **1**: 133-137.
5. **Dahlenborg M, Borch E, Rådström P.** Development of a combined selection and enrichment PCR procedure for *Clostridium botulinum* types B, E, and F and its use to determine prevalence in fecal samples from slaughtered pigs. *Applied and Environmental Microbiology* 2001; **10**: 4781-4788.
6. **Dahlenborg M, Borch E, Radström P.** Prevalence of *Clostridium botulinum* types B, E and F in faecal samples from Swedish cattle. *International Journal of Food Microbiology* 2003; **2**: 105-110.
7. **Notermans S, Dufrenne J, Oosterom J.** Persistence of *Clostridium botulinum* type B on a cattle farm after an outbreak of botulism. *Applied and Environmental Microbiology* 1981; **1**: 179-183.
8. **Heider LC, McClure JT, Leger ER.** Presumptive diagnosis of *Clostridium botulinum* type D intoxication in a herd of feedlot cattle. *Canadian Veterinary Journal* 2001; **42**: 210-212.
9. **Kelch WJ, et al.** Fatal *Clostridium botulinum* toxicosis in eleven Holstein cattle fed round bale barley haylage. *Journal of Veterinary Diagnostic Investigation* 2000; **5**: 453-455.
10. **Galey FD, et al.** Type C botulism in dairy cattle from feed contaminated with a dead cat. *Journal of Veterinary Diagnostic Investigation* 2000; **3**: 204-209.
11. **Notermans S, Kozaki S, van Schothorst M.** Toxin production by *Clostridium botulinum* in grass. *Applied Environmental Microbiology* 1979; **38**: 767-71.
12. **Notermans S.** Control in fruits and vegetables. In Hauschild AHW and Dodds KL. eds. *Clostridium botulinum: Ecology and Control in Foods*. NY, USA: Marcel Dekker Inc; 1993: pp. 239-240.
13. **Martin S.** *Clostridium botulinum* type D intoxication in a dairy herd in Ontario. *The Canadian Veterinary Journal* 2003; **6**: 493-495.
14. **Advisory Committee on the Microbial Safety of Foods.** *Report on botulism in cattle*. London, United Kingdom: United Kingdom Food Standard Agency; 2005.
15. **Steinman A, et al.** Quantitative analysis of levels of serum immunoglobulin G against botulinum neurotoxin type D and association with protection in natural outbreaks of cattle botulism. *Clinical and Vaccine Immunology* 2006; **8**: 862-868.

16. **Gessler F, Hampe K, Böhnelt H.** Sensitive detection of botulinum neurotoxin types C and D with an immunoaffinity chromatographic column test. *Applied Environmental Microbiology* 2005; 71:7897-903. *Immunology and Medical Microbiology* 1996; **4**: 279-285.
17. **Fach P, et al.** Investigation of animal botulism outbreaks by PCR and standard methods. *FEMS Immunology and Medical Microbiology* 1996; **4**: 279-285.
18. **Chaffer M, et al.** Application of PCR for detection of *Clostridium botulinum* type D in bovine samples. *Journal of Veterinary Medicine* 2006; **1**: 45-47.
19. **Prevot V, et al.** Optimization of polymerase chain reaction for detection of *Clostridium botulinum* type C and D in bovine samples. *Zoonoses and Public Health* 2007; **8**: 320-327.
20. **Nordic Committee on Food Analysis.** *Clostridium botulinum*. Detection in foods, blood and other test materials. Method no. 80, 2nd ed. NCFA, Espoo, Finland. 1991.
21. **Lindström M, et al.** Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. *Applied and Environmental Microbiology* 2001; **12**: 5694-5699.
22. **Williamson JL, Roche TE, Aiken JM.** In situ detection of the *Clostridium botulinum* type C1 toxin gene in wetland sediments with a nested PCR assay. *Applied and Environmental Microbiology* 1999; **7**: 3240-3243.
23. **Szabo EA, Pemberton JM, Desmarchelier PM.** Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. *Applied and Environmental Microbiology* 1993; **9**: 3011-3020.
24. **Hyytiä E, Björkroth J, Hielm S, Korkeala H.** Characterization of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. *International Journal food Microbiology* 1999; **48**:179-189.
25. **Keto-Timonen R, et al.** Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. *Journal of Clinical Microbiology* 2006; **11**: 4057-4065.
26. **Nordic Committee on Food Analysis.** Botulinum toxin. Detection in foods, blood and other test materials. Method no. 79, 2nd ed. NCFA, Espoo, Finland. 1991.
27. **Centers for Disease Control and Prevention.** *Clostridium botulinum* monovalent and polyvalent antitoxins. Georgia, USA: U.S. Department of Health, Education, and Welfare; 1987
28. **Lindström M, Korkeala H.** Laboratory diagnostics of botulism. *Clinical Microbiology Reviews* 2006; **2**: 298-314.
29. **Allison MJ, Maloy SE, Matson RR.** Inactivation of *Clostridium botulinum* toxin by ruminal microbes from cattle and sheep. *Applied and Environmental Microbiology* 1976; **5**: 685-688.
30. **Neill SD, McLoughlin MF, McIlroy SG.** Type C botulism in cattle being fed ensiled poultry litter. *The Veterinary Record* 1989; **21**: 558-560.

31. **Segner WP, Schmidt CF, Boltz JK.** Enrichment, isolation, and cultural characteristics of marine strains of *Clostridium botulinum* type C. *Applied Microbiology* 1971; **6**: 1017-1024.
32. **Virtanen, AI.** The A-I-V-method of preserving fresh fodder. *Empire Journal of Experimental Agriculture*, 1933; **1**: 143-155.
33. **Silage Fermentation and Preservation. North Dakota State University.** (<http://www.ag.ndsu.edu/pubs/ansci/dairy/as1254w.htm>). Accessed 27 November 2007.
34. **Lindström M, et al.** Type C botulism due to toxic feed affecting 52,000 farmed foxes and minks in finland. *Journal of Clinical Microbiology* 2004; **10**: 4718-4725.
35. **Huss HH.** Distribution of *Clostridium botulinum*. *Applied and Environmental Microbiology* 1980; **4**: 764-769.
36. **Mylykoski J, et al.** The detection and prevalence of *Clostridium botulinum* in pig intestinal samples. *International Journal of Food Microbiology* 2006; **2**: 172-177.
37. **Otter A, et al.** Risk of botulism in cattle and sheep arising from contact with broiler litter. *The Veterinary Record* 2006; **6**: 186-187.
38. **Siegel, LS.** Destruction of botulinum toxins in food and water. In: AHW Hauschild and KL Dodds (eds.) *Clostridium botulinum. Ecology and Control in Foods*. NY, USA: Marcel Dekker Inc; 1993: pp. 323-341.

Table 1. Clinical and post-mortem findings in the type C bovine botulism outbreak.

Conditions observed ^a	No. of affected (examined) cattle
Clinical findings	
Anorexia	9(9)
Ataxia	9(9)
Apathy	9(9)
Weakness	9(9)
Dysphagia	9(9)
Increased salivation	9(9)
Paralysis, recumbency	9(9)
Dropping of feed pieces from the mouth	9(9)
Decreased ruminal contractions	7(9)
Tail paralysis	3(9)
Increased ruminal pH	2(2)
Flaccid paralysis of tongue	2(2)
Paralysis of jaw muscles	2(2)
Dry faeces	1(9)
Diarrhoea	1(9)
Post-mortem findings	
Aspiration pneumonia	1(3)
Mucosal hyperaemia in abomasum, duodenum and jejunum	2(2)
Petechial haemorrhages in rumen and omasum	2(2)
Centrilobular hepatic degeneration	2(2)

^a Anorexia and ataxia were the first clear signs observed

Table 2. Results of analyses on three bovine samples and silage for pH, botulinum neurotoxin gene types A-F, presence and type of botulinum neurotoxin and isolation of *C. botulinum*

Animals/sample material	pH	PCR for botulinum neurotoxin genes						Mouse bioassay No. of positive samples (no. of samples tested)	Type	Isolation (no. [type] of isolates)
		A	B	C	D	E	F			
Heifer no. 3										
Intestinal wall	ND ^a	-	-	+	-	-	-	ND		0
Intestinal content	7.43	-	-	+	-	-	-	2(2)	C	0
Abomasal content	5.73	-	-	+	-	-	-	2(2)	C	0
Liver	6.32	-	-	+	-	-	-	2(2)	C	2 ^b (C)
Cow no. 3										
Intestines	ND	-	-	-	-	-	-	ND		0
Intestinal content	5.84	-	-	-	-	-	-	0(2)		0
Ruminal content	8.04	-	-	-	-	-	-	0(2)		0
Cow no. 4										
Ruminal content	7.72	-	-	-	-	-	-	ND		0
Faeces	6.89	-	-	-	-	-	-	ND		0
Blood	ND	ND	ND	ND	ND	ND	ND	0(2)		0
Silage	5.97	-	-	-	-	-	-	0(2)		0

^a Not determined

^b AFLP analysis revealed the two isolate profiles to be identical

Table 3. Comparison of the effect of pretreatments on PCR detection of *C. botulinum* neurotoxin gene from samples of heifer no. 3. Samples were cultured in TPGY broth, and on EYA agar and blood agar plates, prepared with (E) and without (NE) ethanol treatment (NCFA 1991), and incubated at 30°C and 37°C.

Temp	Liver						Abomasal content						Intestinal content						Intestinal wall					
	TPGY		EYA		Blood		TPGY		EYA		Blood		TPGY		EYA		Blood		TPGY		EYA		Blood	
	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE
30°C	+	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	ND ^a	ND	ND	ND
37°C	+	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	ND	ND	ND	ND

^a ND, not determined

LEGENDS FOR FIGURES

Fig.1. AFLP dendrogram comparing the outbreak *C. botulinum* type C strains CBO1C-1 and CBO1C-2 with a *C. botulinum* type C strain from the library at the DFEH. The outbreak strains clustered in *C. botulinum* Group III and had a similarity of 84% with the Stockholm C strain. The clostridial strains used are described by Keto-Timonen *et al.* (2006).