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Clostridium botulinum type D/C intoxication in a dairy cow stock in Saxony-Anhalt (Germany) – report on an innovative diagnostic approach

Clostridium botulinum Typ D/C Intoxikation einer Milchviehherde in Sachsen-Anhalt (Deutschland) – Vorstellung einer innovativen diagnostischen Vorgehensweise

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Summary

Botulism in cattle is a rare but serious disease. In Germany there is no obliga-
tion to report botulism in animals and therefore a precise morbidity rate is not
available. In this manuscript we describe an outbreak of *Clostridium* (*C.*) *botuli-
num* neurotoxin (BoNT) intoxication in a Saxony-Anhalt dairy cow stock of 286
Holstein-Friesian cows and offspring in spring/summer 2009 and its diagnostic
approach. 122 animals showed clinical signs of BoNT intoxication. 115 of the
affected animals (40.2% of the herd) independent of age died or had to be eutha-
nized. Therapeutic attempts failed in almost all diseased cows, only four calves
and three heifers recovered. Diagnostic samples of several animals (n = 4) (liver,
ruminal and intestinal contents) and feed (n = 6) were tested for BoNT genes by
polymerase chain reaction (PCR). BoNT gene type D was found in several
(n = 8) organ samples. The PCR results allowed a preselection of samples for BoNT
that were then tested by the mouse bioassay. Thus, the number of mice being
inoculated in the mouse bioassay could be reduced. The mouse bioassay turned
out positive (wasp-waist) in three preselected organ samples and the neutraliza-
tion test of one sample with type-specific antitoxin confirmed the presence of
BoNT type D. We succeeded in isolating a *C. botulinum* strain from a liver sample
which was typed as a D/C mosaic strain by sequence analysis of the toxin gene.
However, the source of the BoNT intoxication could not be traced back.

Keywords: Botulism, cattle, *Clostridium botulinum* type D/C, PCR, mouse bioassay

Zusammenfassung

Botulismus bei Rindern ist eine seltene, aber schwerwiegende Erkrankung. In
Deutschland besteht keine Melde- oder Anzeigepflicht für Botulismus, sodass
keine Daten zur Erkrankungshäufigkeit verfügbar sind. Im vorliegenden Fallbe-
richt wird ein Botulismus-Ausbruch in einem Milchviehbetrieb in Sachsen-Anhalt
beschrieben. Der Fall ereignete sich im Frühjahr/Sommer 2009 in einem Betrieb
mit insgesamt 286 Holstein-Rindern. Bei 122 Tieren fielen klinische Anzeichen
einer Intoxikation mit *Clostridium* (*C.*) *botulinum* Neurotoxin (BoNT) auf. 115 der
klinisch erkennbar betroffenen Tiere (40,2 % des Bestandes) aller Altersgruppen
verendeten oder mussten euthanasiert werden. Therapieversuche blieben meist

erfolglos, lediglich vier Kälber und drei Färsen erholten sich von der Erkrankung. Verschiedene Organproben von mehreren Tieren (n = 4) (Leber, Panseninhalt und Darminhalt) und Futtermittel (n = 6) wurden mittels Polymerase-Kettenreaktion (PCR) untersucht. BoNT Typ D Gensequenzen waren in mehreren Organproben (n = 8) nachweisbar. Diese Ergebnisse erlaubten eine Vorauswahl von Proben, die nachfolgend mit dem Maus-Bioassay untersucht wurden, wodurch die Anzahl der benötigten Versuchstiere reduziert werden konnte. Der Maus-Bioassay erbrachte bei den 3 vorausgewählten Proben einen positiven Neurotoxin-Nachweis (Wespentaille). Der Neutralisationstest einer ausgewählten Probe mit typspezifischen Antitoxinen bestätigte die Anwesenheit von BoNT Typ D. Aus einer Leberprobe gelang die Isolierung eines *C. botulinum*-Stammes. Durch Sequenzierung des BoNT Gens konnte eine BoNT D/C Mosaikform des Toxin-Gens nachgewiesen werden. Die Eintragsquelle des Toxins konnte nicht ermittelt werden.

Schlüsselwörter: Botulismus, Rind, *Clostridium botulinum* type D/C, PCR, Maus-Bioassay

Introduction

Botulism is a disease caused by *Clostridium* (*C.*) *botulinum*, a Gram-positive spore forming anaerobic bacterium which secretes a highly potent neurotoxin (Rossetto et al. 2014). *C. botulinum* neurotoxin (BoNT) blocks the neurotransmitter release in peripheral nerve endings causing muscular paralyses. These neurotoxins and the corresponding *C. botulinum* isolate are characterised using a serotype system. Seven serotypes i. e. A, B, C, D, E, F and G are currently known, while a proposed novel serotype remains to be verified (Rossetto et al., 2014). Botulism in cattle is most often caused by *C. botulinum* serotypes C and D. Clinical symptoms usually start after uptake of feed containing the toxin (Lindström et al., 2010). BoNT is absorbed via the intestines. It is then via the bloodstream transported to the neuromuscular junctions, where it inhibits the secretion of acetylcholine and generally leads to neuro-paralytic disease (Smith and Sugiyama, 1988). Clinical symptoms of botulism in cattle are depending on the dose and type of toxin ingested. Signs of botulism can dramatically vary from sudden death (animal collapse in a couple of hours) to slowly progressive paralysis with animals dying after days. Affected cattle often develop an unstable gait and eventually become recumbent. Typical symptoms are also cumulative chewing and swallowing disorders, profuse loss of saliva and a reduced tongue tonus which complicates feed and water uptake (Braun, 2006). Paralysis of the diaphragm and the intercostal muscles causes aggravated breathing and finally death. Paralysis progressing from cranial to caudal was described as well as paralysis starting at the hind legs (Braun, 2006; Haagsma, 1991). Caudal to cranial paralysis was described to start with instable movements, extended resting and difficulties to rise, followed by hind limb paralysis progressing to the forelegs, neck and head. A reduced tone of the tail can be remarkable. In the later course of the disease breathing difficulties, recumbency and finally death was observed. Some authors label this course of disease as atypical (Haagsma, 1991). A broad range of differential diagnoses has to be considered including infectious and metabolic diseases and toxaemia (Braun, 2006; Haagsma, 1991). Botulism is commonly diagnosed by proof of toxin from clinical samples, such as rumen or intestinal contents using the mouse bioassay (Lindström and Korkeala, 2006). The mouse bioassay is still the standard diagnostic

FIGURE 1: *Cow showing clinical signs of sternal recumbency and hind limb weakness.*

procedure for the detection of biologically active BoNT in clinical samples and the final proof for causality. The mouse bioassay is very sensitive and specific and allows the detection of any BoNT serotype in a wide variety of clinical samples (Dorner et al., 2013; Lindström and Korkeala, 2006). Depending on the protocol used for the detection of toxicity, a minimum of six animals per sample are needed, if serotyping is conducted another six animals will be required for each serotype (CDC, 1998; Solomon and Lilly, 2001; L06.00-26 [DIN 10102] of the official collection of analysis methods according to § 64 of the German Food and Feed Code [LFGB]).

In this case report, we applied screening PCR to reduce the number of animals required in the current standard test, the mouse bioassay. *C. botulinum* neurotoxin type D was shown to be the cause of disease.

Case description and diagnostic procedures

Case description

In May 2009 various animals of a dairy cow stock in Saxony-Anhalt (Germany) with 286 Holstein cows and offspring showed clinical signs of a paralytic disease, thus *C. botulinum* intoxication was suspected. The first dead animal was noticed on May 12th. Cases occurred in a slightly
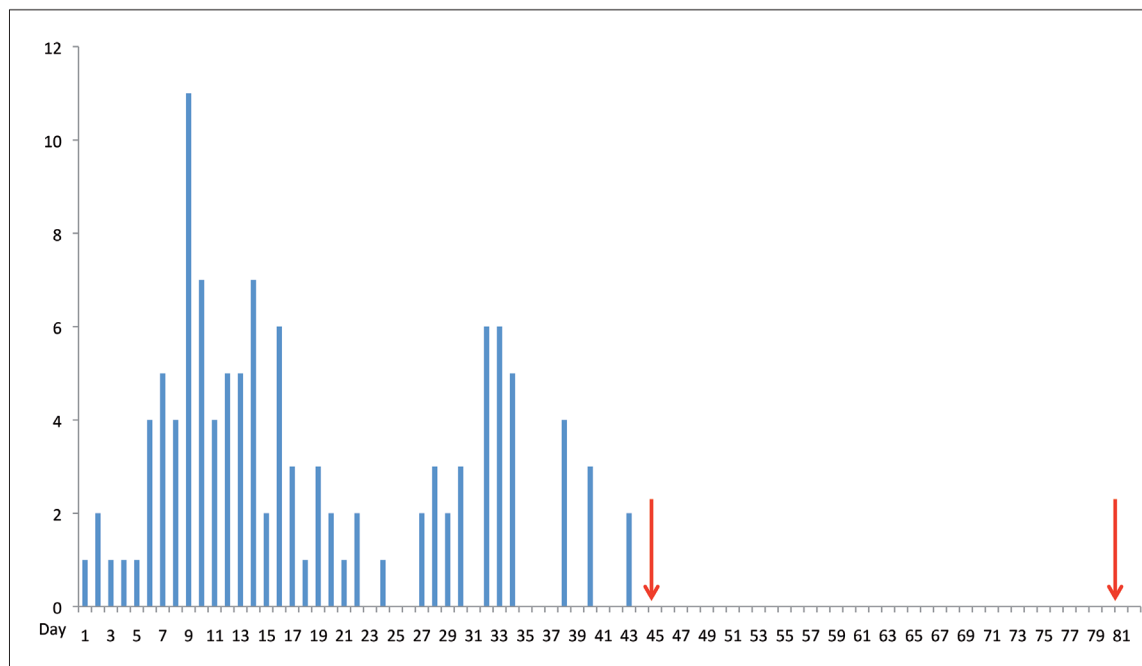
FIGURE 2: *Course of the outbreak of botulism in cattle. The blue columns show the number of dead animals each day. Day 1 is the start of the outbreak when the first dead animal was noticed. The red arrows indicate vaccination. After the first application on day 45 of the outbreak the second vaccination was administered 36 days later.*

biphasic manner (Fig. 2) till end of June, with a decrease at the end of May and beginning of June. 115 of 122 diseased cows (40.2% of the stock) died or had to be euthanized. Age distribution and numbers of affected animals are listed in Table 1. Death of cows in late gravidity happened almost always shortly after being dried off. Deaths of cows in early stage of lactation were noticed in the later course of the events. Therapeutic attempts (see subsequent paragraph) failed in all cows and only four calves and three heifers recovered. Affected cows showed progressive caudal to cranial paralysis, starting with hind limb ataxia which in severe cases deteriorated to recumbency (Fig. 1). Additionally, laboured breathing, ataxia, staggered rising and a reduced tongue tonus were observed. In the majority of cases, consciousness, appetite as well as food intake seemed undisturbed. Approximately 5% of all cases showed a peracute course of disease with animals developing sudden recumbency after the last milking with a normal milk yield or sudden death without clinical signs. About 10% of all cases showed slow progression with clinical signs developing over ten to 14 days with final recumbency. Calves and heifers showed the same clinical signs and comparable development of disease, but in acute cases a fast progressing cachexia was additionally noted. Approximately 40% of the affected calves (four of eight) and heifers (three of nine) recovered even after some of them having been recumbent for one up to 14 days.

Therapeutic approach
Animals were treated symptomatically with parenteral administration of fluids and anti-inflammatory drugs. Vaccination of all animals with *C. botulinum* vaccine (Botulism vaccine for cattle, horses, mules, sheep and goats, Onderstepoort Biological Products SOC Ltd, Onderstepoort, South Africa) was applied according to the manufacturers' instructions. After the first application on day 45 of the outbreak (Fig. 2) no more clinical cases occurred. The second vaccination was administered 36 days later.

Sample collection
Mid-May, four cattle cadavers were directed to the pathology laboratory of the Saxony-Anhalt State Office for Consumer Protection, Department 4 – Veterinary Medicine, Stendal for gross pathology. Organ material (ruminal content, intestinal content, liver) and feed stuff (feed 1–5: milking cows' mixed ration, maize grain silage, sugar-beet pressed pulps, wilted grass silage, maize silage) were passed on to the Institute of Bacterial Infection and Zoonoses, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Jena.

In an attempt to identify the source of intoxication, one month after testing the samples mentioned above, a farm visit was undertaken and further samples were collected including feed (feed 6: maize grain silage), water, a pigeon corpse, pigeon faeces (pigeons were present at the farm and in the stables at higher numbers) and chicken faeces (a poultry farm was located nearby the affected stables). The precise numbers and sort of samples investigated are shown in Table 3.

Mouse bioassay
To prepare the extract for the mouse bioassay, 80 g of each sample was mixed with an equal volume (80 ml) of gelatine-phosphate buffer (2 g gelatine, 4 g disodium hydrogen phosphate, 1000 ml aqua dest., pH = 6,2 ± 0,1) using a Stomacher® 400 circulator (250 rpm, 1 min; Seward Ltd., Worthing, UK). An exception was made concerning wilted grass silage, wherefore only 40 g were mixed with 80 ml of gelatine-phosphate buffer. This preparation was incubated in a refrigerator overnight at 4°C. The liquid content was transferred to a centrifuge tube (Nalgene; Thermo Fisher Scientific Inc. Waltham, MA., USA) and centrifuged for 20 min at 10 000 rcf (xg) at 4°C (Biofuge 22R-Heraeus, Offenbach, Germany). The maintained supernatant was then filtered (0.22 µm syringe filter, Sartorius, Göttingen, Germany) to ensure sterility.

The resulting extract was tested for the presence of botulinum neurotoxin by the mouse bioassay. Adult male and female BALB/c mice (18–25 g) were used in

the mouse bioassay. The mouse bioassay and treatment of extracts followed method L06.00-26 (DIN 10102) of the official collection of analysis methods according to § 64 of the German Food and Feed Code (LFGB). Boiled extracts (100°C for ten min) were used as control samples. Trypsin treated extracts, diluted 1:2 in gelatine-phosphate buffer containing 1% trypsin (Trypsin (1:250), powder, Life Technologies GmbH, Darmstadt, Germany), and the non-trypsin treated extracts, diluted 1:2 in gelatine-phosphate buffer, were prepared. Two mice were injected with 0.5 ml of each extract (extract, trypsin treated extract, heated extract), respectively, to determine toxicity. Trypsin treated samples were omitted in the mouse bioassay, if the proteolytic activation of BoNT was not required (i. e. for type C and D) (Cook et al., 1998; Lindström and Korkeala, 2006).

Toxin typing by neutralization assay was performed with type specific monovalent antitoxin sera type A to F (CIDC Lelystad, NL) according to method L06.00-26. Where appropriate, antisera were pooled for the mouse bioassay, i. e. as BoNT type D gene was detected by PCR, non-relevant antisera types A, B, E and F were pooled. Mice were observed for four days. The extract dilutions 1:2, 1:20 and 1:200 of the caecum sample of cow 2 were subjected to the neutralization mouse bioassay. One group of mice (n = 6) received the extract without antiserum, another group received the extract including the pooled antisera type A, B, E and F, the next group received the extract including antiserum type C and the last group received the extract including antiserum type D. The mouse bioassay was performed according to the German Animal Welfare Act with permission of the respective authorities (Thuringia, State Office for Consumer Protection, reg. no. 22-2684-04-04-104/07).

DNA extraction

DNA extraction of each sample was performed from the overnight extraction mixture (80 g sample plus 80 ml gelatine-phosphate buffer) for the mouse bioassay before it was centrifuged. 1.5 ml of the mixed sample was removed and centrifuged at 8000 g for ten min. The resulting pellet was used for DNA extraction with DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for Gram positive bacteria.

For DNA extraction after an enrichment step, each sample was additionally brought in liquid culture medium and incubated anaerobic at 37°C for 6–11 days. A total of either 1 g of sample material or 2 g of mixed sample (see above) was inoculated into 10 ml of either reinforced clostridial medium (RCM, Oxoid, Wesel, Germany) or modified cooked meat medium containing 1.5 g meat particles (cooked meat medium, Oxoid, Wesel, Germany) and 15 ml nutrient solution containing 7.8 g casein peptone, 7.8 g meat peptone, 1.0 g glucose, 2.8 g yeast extract, 1.0 g soluble starch, 5.6 g sodium chloride per litre. Then 1.5 ml was removed and centrifuged at 8000 g for 10 min. The resulting pellet was used for DNA extraction with DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as described.

BoNT gene PCR

Detection of *C. botulinum* type C and D neurotoxin (BoNT type C and D) genes was done by single PCR described elsewhere (Takeshi et al., 1996). Briefly, oligonucleotide primers (MWG Eurofins, Ebersberg, Germany) for the detection of BoNT type C and D genes were used as described (Takeshi et al., 1996). BoNT type C primer CS-11: 5´-ATA CAC TAG CTA ATG AGC CTG-3´ and CS-22: 5´-TGG AGT ATT GTT ATT CCC AGG-3´. BoNT type D primer DS-11: 5´-GTG ATC CTG TTA ATG ACA ATG-3´ and DS-22: 5´-TCC TTG CAA TGT AAG GGA TGC-3´ amplification products are 290 bp for BoNT type C gene and 497 bp for BoNT type D gene. A total of 25 µl PCR reaction volume was used, a reaction contained 5 pmol of each primer, 250 µM dNTP mix, 1 mM MgCl$_2$, 1x PCR Buffer (Qiagen, Hilden, Germany), 1x Q-Solution (Qiagen, Hilden, Germany), 1.25 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Amplification was carried out in a thermal cycler (Mastercycler, Eppendorf AG, Eppendorf, Germany). PCR reaction was subjected to the following conditions: initial denaturation for 15 min at 95°C; 35 cycles of denaturation (15 sec at 95°C), annealing (one min at 55°C), extension (one min at 72°C); and a final extension at 72°C for 3 min. PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, visualized with UV illumination at 312 nm and finally photo documented.

Isolation of *C. botulinum*

One cm³ of a PCR BoNT type D gene positive liver sample was kept in methylated spirit for five days and then streaked out on egg-yolk agar plates (Columbia Agar Base (Oxoid, Wesel, Germany) containing 10% Egg Yolk Emulsion (Oxoid), 1% Lactose and 1% of a 0,4% Bromocresol purple solution (Merck, Darmstadt Germany) which were previously stored under anaerobic conditions. The plates were incubated at 37°C for 24 to 48 h under anaerobic conditions. Single colonies showing a mother-of-pearl layer and optionally a precipitation zone were subsequently subcultured and tested for BoNT type D gene by PCR (Takeshi et al., 1996).

Sequence analysis of the toxin gene

For sequence analysis, five PCR primer pairs were designed generating overlapping fragments (Tab. 2). Primer sequences were derived from BoNT type D/C chimeric sequence GenBank Accession number D38442.1 (Moriishi et al., 1996). A total of 50 µl PCR reaction volume was used, a reaction contained 10 pmol of each primer, 250 µM dNTP mix, 1.5 mM MgCl$_2$, 1x PCR Buffer (Qiagen, Hilden, Germany), 1.25 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Amplification was carried out in a thermal cycler (Mastercycler, Eppendorf AG, Eppendorf, Germany). PCR reaction was subjected to the following conditions: initial denaturation for 15 min at 95°C; 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 50°C), extension (1 min at 72°C); and final extension at 72°C for 2 min. PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, visualized with UV illumination at 312 nm and finally photo documented. PCR products were purified using High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The PCR products were sequenced by cycle sequencing (Eurofins Genomics GmbH, Ebersberg, Germany) using both, forward and reverse primers. Sequences obtained from both runs were aligned, primer sequences were removed and reading mistakes were corrected. For obtaining the whole toxin gene sequence, overlapping sequences were aligned using Geneious version 8.0.5. (Kearse et al., 2012).

TABLE 1: *Overview of the number of affected animals (diseased and perished/euthanized) according to their age cohort and stock number*

Age cohort	Stock number (11.05.2009)	Animals (state end of June 2009)	
		Diseased	Perished/ Euthanized (%)
Calves	69	8	4 (5,8)
Young cattle/heifers	18	9	6 (33,3)
Cows (early lactation)	25	21	21 (84)
Cows (later lactation)	143	72	72 (50,3)
Cows (dried off, phase 1)*	6	10	10 (100*)
Cows (dried off, phase 2)	25	2	2 (8)
Total number	286	122	115 (40,2)

* Cows of the later lactation group changed to the dried off phase I group, mortality rate cannot exceed 100%

TABLE 2: *Sequencing primers used to determine the nucleotide sequence of the BoNT gene of the outbreak isolate. Primer sequences were selected according to GenBank Accession number D38442.1 strain „South African" (Moriishi et al., 1996)*

Name	Sequence	Nucleotide Position	PCR-Product in bp
F1	5´-TGGATATTAAAAAGTTAGGAGAT-3´	131–153	
R1	5´-GAAGTGGTCCAAATATCAAT-3´	630–649	528
F2	5´-AGTTTGAAAATGGTAGTTGG-3´	581–600	
R2	5´-CAAATCTACTACACTTTCTGA-3´	1438–1458	877
F3	5´-TAATATAGAAAATTCGGGTCAG-3´	1380–1401	
R3	5´-GATAACTTATATGATTAAATTGAGT-3´	2302–2326	946
F4	5´-GAGTTAAGAGATGGAAAGATT-3´	2240–2260	
R4	5´-CATCATATTGGTAGTAATAGTTA-3´	3179–3201	961
F5	5´-AGTGAACAAGATATAAACTTTAGT-3´	3109–3132	
R5	5´-TTTAACATAATTTATACTATTACTTATT-3´	4022–4049	940

bp = basepairs

Results

C. botulinum neurotoxin detection was performed on animal and feed samples from a Saxony-Anhalt dairy cow stock. In this herd the reported clinical picture i. e. paralysis and no specific gross pathological findings was highly suspicious for botulism but the initial examination of samples of cow 1 showed no toxicity in the mouse bioassay. Five days later, samples from cow 2, 3, 4 and feed samples 1–5 arrived at the FLI. The feed samples were immediately prepared for the mouse bioassay to possibly identify a toxin source but no toxicity was detected. In the further diagnostic procedures we investigated the environmental and feed samples by PCR to preselect samples and to avoid additional mouse bioassays (Tab. 3).

PCR investigation of organ samples of cow 1 was performed. A DNA extract from a small intestine sample of cow 1 tested positive for BoNT type D gene (Tab. 3). Samples from liver and rumen content as well as the enriched samples of gut, liver and rumen contents tested negative for BoNT type C and D genes (Tab. 3). Also direct extraction based PCRs as well as enrichment culture based PCRs of feed samples 1–5 tested negative for BoNT type C and D genes. All environmental samples tested negative in the PCR assay (Tab. 3).

In order to rapidly select samples for the mouse bioassay, PCR was performed on DNA samples from cows 2, 3 and 4 omitting a pre-enrichment step. BoNT type D gene DNA was detected in the caecum samples of cow 2 and 3 and in a liver sample of cow 4 (Tab. 3). Based on these results it was decided to test the three PCR positive

samples in the mouse bioassay (Tab. 3). All extracts investigated tested toxic to mice, toxicity signs detected were indicative for BoNT. The corresponding heated extracts used as negative-controls tested non-toxic. Based on the PCR (BoNT type D gene) and the respective mouse bioassay results, it was decided to test one sample in the neutralization assay.

For this purpose the caecum sample of cow 2 was selected, whose extract dilutions including the pooled antisera A, B, E and F and extract dilutions including the antiserum C tested toxic. All mice receiving extract dilutions including the antiserum type D survived and showed no symptoms.

C. botulinum was isolated from liver tissue of cow 4 (Tab. 3). The isolate tested positive for BoNT type D gene by PCR (Takeshi et al., 1996). Subsequent initial sequencing of the BoNT gene resulted in an incomplete BoNT type D/C chimeric sequence (Data not shown). Sequencing was repeated using primers derived from an available BoNT type D/C sequence (GenBank Accession number D38442.1) (Tab. 2). We were able to obtain a 3879 bp sequence containing the complete coding sequence of a chimeric BoNT type D/C gene (Accession number KT025245).

Discussion

Our cumulative data give evidence that the disease causing the death of 40.2% of the dairy cow stock on the affected farm was caused by *C. botulinum* intoxication. This report is an example for a possible strategy for laboratory diagnosis of a botulism outbreak using a combination of PCR and mouse bioassay in order to reduce the number of mice needed for definite diagnosis. Basically, the preliminary clinical diagnosis and gross pathology suggested botulism. Our focus was to examine selected animal samples to confirm the clinical diagnosis and to identify the BoNT serotype. Feed and environmental samples were analysed to possibly trace back the source of this botulism outbreak.

The first therapeutic approach in suspected botulism would be the administration of polyvalent antitoxin as soon as possible because it is effective only against circulating toxin and not effective when the toxin has reached the neuromuscular junction and entered the nerve terminus (Anniballi et al., 2013). However, polyvalent antitoxin usually produced for use in humans is very expensive and not available for the treatment of cattle botulism. Furthermore, these polyvalent sera formulations usually do not contain antitoxin type C and D. Consequently, the initial therapeutic approach was not to use anti-toxin but concentrated on supporting measures against metabolic stress and stabilisation of metabolism. Also anti-inflammatory drugs were administered. However, reduced water and food intake must be addressed therapeutically when it is observed. Vaccination is commonly used as a preventive measure in areas where botulism is endemic. It is also considered an effective approach in outbreak situations (Anniballi et al., 2013). We decided to apply vaccination as no source of intoxication could be found and the outbreak continued. Vaccination had to be permitted by the local health authorities because no commercial vaccine is licensed in Germany. Appropriate diagnosis of the serotype is required for the selection of the vaccine. A vaccine (Botulism Vaccine, Onderstepoort Biological

Products SOC Ltd, Onderstepoort, South Africa) which contains type C and type D toxoid was used. Finally we cannot state whether the vaccination had an influence on the termination of the outbreak at all or if simply the toxin uptake ceased e. g. contaminated feed was totally consumed. Still we would recommend emergency vaccination and if possible replacement of feedstuff.

Laboratory diagnosis of botulism is challenging because of the extraordinary toxic potency and variability of BoNT. Despite a variety of promising developments in detection technologies for BoNT e. g by so called Endopep MS (Dorner et al., 2013; Kalb et al., 2015; Singh et al., 2013) the mouse bioassay still is the standard diagnostic procedure for the detection of biologically active BoNT in clinical samples and the final proof for causality (Dorner et al., 2013; Lindström and Korkeala, 2006; Singh et al., 2013). Initially, samples from one animal reached the laboratory and were immediately investigated for the presence of BoNT. Unfortunately, the performed mouse bioassay failed to detect toxins in the tested samples. Similar findings were reported in former botulism outbreaks, where it was difficult to detect BoNT at all (Abbitt et al., 1984; Sharpe et al., 2008; Trueman et al., 1992). A negative mouse bioassay does not exclude botulism, because toxin may not be present in the selected samples at a detectable level. Therefore, more samples have to be tested. We decided to primarily screen the majority of samples by PCR and to follow up only those showing a positive PCR result. That pre-selection of samples facilitated a significant reduction of mice required to perform the mouse bioassay. Additionally, trypsin treated samples were omitted in the mouse bioassay because the proteolytic activation of BoNT type D in samples is not required (Cook et al., 1998; Lindström and Korkeala, 2006), which allowed a further saving of mice. Our modified protocol allowed us to test three samples using four mice each instead of nine samples (organ samples of cow 2, 3, and 4) with six mice each, saving 42 mice compared to the standard protocol. The mouse bioassay tested positive (wasp-waist) in all organ samples chosen. The neutralization test with type D specific antitoxin of one selected sample confirmed the presence of BoNT type D. Cross neutralization effects with type C specific antitoxin were not observed. In summary, the approach specified above effected a saving of mice, time and money.

The PCR used for detection of BoNT genes (Takeshi et al., 1996) targets a conserved region within the first third of the coding sequence. Sequence analysis revealed sequence similarity to a chimeric form of BoNT type D/C genes previously described to cause botulism in cattle in Japan (Nakamura et al., 2010) and in Europe (Woudstra et al., 2012). These BoNT D/C types are highly represented among European bovine isolates (Woudstra et al.,

TABLE 3: *Comparison of results of toxin gene detection by PCR using DNA extractions from samples (direct extraction) and DNA extractions from enrichment culture and the results of detection of toxicity and toxin typing by mouse bioassay*

Sample		Number	PCR				Mouse bioassay	
			direct extraction		enrichment culture		toxicity	typing
			type C	type D	type C	type D		
Cow 1	rumen	1	neg.	neg.	neg.	neg.	neg.	-
	small intestine	1	neg.	pos.	neg.	neg.	neg.	-
	liver	1	neg.	neg.	neg.	neg.	neg.	-
Cow 2	rumen	1	neg.	neg.	-	pos.	-	-
	caecum	1	neg.	pos.	-	pos.	pos.	type D
	liver	1	neg.	neg.	-	neg.	-	-
Cow 3	rumen	1	neg.	neg.	-	pos.	-	-
	caecum	1	neg.	pos.	-	pos.	pos.	-
	liver	1	neg.	neg.	-	pos.	-	-
Cow 4	rumen	1	neg.	neg.	-	pos.	-	-
	caecum	1	neg.	neg.	-	neg.	-	-
	liver	1	neg.	pos.	-	pos.	pos.	-
Feed 1	milking cows mixed ration	1	neg.	neg.	-	neg.	neg.	-
Feed 2	maize grain silage	1	neg.	neg.	-	neg.	neg.	-
Feed 3	pressed sugar beet pulps	1	neg.	neg.	-	neg.	neg.	-
Feed 4	wilted grass silage	1	neg.	neg.	-	neg.	neg.	-
Feed 5	maize silage	1	neg.	neg.	-	neg.	neg.	-
Feed 6	maize grain silage	2	neg.	neg.	neg.	neg.	-	-
Water		1	neg.	neg.	neg.	neg.	-	-
Pigeon	faeces	5	neg.	neg.	neg.	neg.	-	-
Pigeon	small intestine	1	neg.	neg.	neg.	neg.	-	-
Pigeon	caecum	1	neg.	neg.	neg.	neg.	-	-
Pigeon	colon	1	neg.	neg.	neg.	neg.	-	-
Chicken	faeces	1	neg.	neg.	neg.	neg.	-	-

pos. = positive

neg. = negative

- = not investigated

2012). Indeed, this is the first case report of mosaic gene BoNT type D/C cattle botulism in Germany. The chimeric form of BoNT type D/C was completely neutralized by type D antiserum. In contrast the antitoxin type C did not protect the mice; similar findings were previously reported (Nakamura et al., 2010). Historic underreporting of chimeric BoNT type D/C in cattle seems well probable.

Environmental contamination might be a source for the described botulism outbreak. Type C botulism in cattle has been previously reported in association with a botulism outbreak in wild waterfowl (Wobeser et al., 1997). Silage contaminated with poultry carcasses caused botulism in cattle (Hogg et al., 1990). Water was also implicated in botulism outbreaks (Jean et al., 1995; Swift et al., 2000; Wobeser et al., 1997). Even though we were not able to trace back the outbreak, we cannot exclude an environmental source of the botulism intoxication. Similar results were previously reported, where even with the mouse bioassay no BoNT was detected in the animal and feed samples at a dairy farm in the Republic of Ireland, although the history, clinical signs and post-mortem examination strongly suggested botulism (Sharpe et al., 2008). Moreover various botulism outbreaks in England and Wales were associated with poultry litter despite the source remained unknown because routine examination of broiler litter and feed were not undertaken (Payne et al., 2011).

The source of the botulinum neurotoxin intoxication of the dairy cow stock could not be identified but close local and organizational connection to poultry was present, which is considered a risk factor (Payne et al., 2011).

Conflict of interest

The authors declare that there is no conflict of interests.

References

Abbitt B, Murphy MJ, Ray AC, Reagor JC, Eugster AK, Gayle LG, Whitford HW, Sutherland RJ, Fiske RA, Pusok J (1984): Catastrophic death losses in a dairy herd attributed to type D botulism. J Am Vet Med Assoc 185: 798–801.

Anniballi F, Fiore A, Lofstrom C, Skarin H, Auricchio B, Woudstra C, Bano L, Segerman B, Koene M, Baverud V, Hansen T, Fach P, Tevell Aberg A, Hedeland M, Olsson Engvall E, De Medici D (2013): Management of animal botulism outbreaks: from clinical suspicion to practical countermeasures to prevent or minimize outbreaks. Biosecur Bioterror. 11 Suppl 1: S191–199.

Braun U (2006): [Botulism in cattle]. Schweiz Arch Tierheilkd 148: 331–339.

Centers for Disease Control and Prevention (CDC) (1998): Botulism in the United States, 1899–1996. Handbook for Epidemiologists, Clinicians, and Laboratory Workers, Atlanta, GA. Centers for Disease Control and Prevention, 1998.

Cook LV, Lee WH, Lattuada CP, Ransom GM (1998): Chapter 14. Methods For The Detection Of *Clostridium Botulinum* Toxins In Meat And Poultry Products. In: Dey BP, Lattuada CP, Mcnamara AM, Mageau RP, and Green SS (eds.), Microbiology Laboratory Guidebook. United States Department Of Agriculture, Food Safety And Inspection Service Office Of Public Health And Science, Microbiology Division, Washington, DC.

Dorner MB, Schulz KM, Kull S, Dorner BG (2013): Complexity of botulinum neurotoxins: challenges for detection technology. Curr Top Microbiol Immunol 364: 219–255.

Haagsma J (1991): [Botulism in cattle, a review]. Tijdschr Diergeneeskd 116(13): 663–669.

Hogg RA, White VJ, Smith GR (1990): Suspected botulism in cattle associated with poultry litter. Vet Rec 126: 476–479.

Jean D, Fecteau G, Scott D, Higgins R, Quessy S (1995): *Clostridium-botulinum* type-C intoxication in feedlot steers being fed ensiled poultry litter. Can Vet J 36: 626–628.

Kalb SR, Krilich JC, Dykes JK, Luquez C, Maslanka SE, Barr JR (2015): Detection of botulinum toxins A, B, E, and F in foods by Endopep-MS. J Agric Food Chem 63: 1133–1141.

Kearse M, Moir R, Wilson A, Stones-Havas, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A. (2012): Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649.

Lindström M, Korkeala H (2006): Laboratory diagnostics of botulism. Clin Microbiol Rev 19: 298–314.

Lindström M, Myllykoski J, Sivela S, Korkeala H (2010): *Clostridium botulinum* in cattle and dairy products. Crit Rev Food Sci Nutr 50: 281–304.

L06.00-26: Nachweis von *Clostridium botulinum* und *Botulinum-Toxin* in Fleisch und Fleischerzeugnissen (nach DIN 10102); Amtliche Sammlung von Untersuchungsverfahren nach § 64a LFGB, BGBl Jhg. 2006 Teil I Nr. 20 vom 26.04.2006 (vormals § 35 LMBG); Beuth Verlag GmbH Berlin Wien Zürich 2004; Band 1/2; Lebensmittel; Teil 2.

Moriishi K, Koura M, Abe N, Fujii N, Fujinaga Y, Inoue K, Ogumad K (1996): Mosaic structures of neurotoxins produced from *Clostridium botulinum* types C and D organisms. Biochim Biophys Acta 1307: 123–126.

Nakamura K, Kohda T, Umeda K, Yamamoto H, Mukamoto M, Kozaki S (2010): Characterization of the D/C mosaic neurotoxin produced by *Clostridium botulinum* associated with bovine botulism in Japan. Vet Mic 140: 147–154.

Payne JH, Hogg RA, Otter A, Roest HI, Livesey CT (2011): Emergence of suspected type D botulism in ruminants in England and Wales (2001 to 2009), associated with exposure to broiler litter. Vet Rec 168: 640.

Rossetto O, Pirazzini M, Montecucco C (2014): *Botulinum* neurotoxins: genetic, structural and mechanistic insights. Nat Rev Microbiol 12: 535–549.

Sharpe AE, Brady CR, Byrne W, Moriarty J, ONeill P, McLaughlin JG (2008): Major outbreak of suspected botulism in a dairy herd in the Republic of Ireland. Vet Rec 162: 411–414.

Singh AK, Stanker LH, Sharma SK (2013): *Botulinum* neurotoxin: where are we with detection technologies? Crit Rev Microbiol 39: 43–56.

Smith LDS, Sugiyama H (1988): Botulism. The organism, its toxins, the disease Charles C. Thomas Publisher, Springfield, IL.

Solomon HM, Lilly T (2001): Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum*, U.S. Food and Drug Administration http://www.fda.gov/Food/ScienceResearch/Laboratory Methods/BacteriologicalAnalyticalManualBAM/default.htm

Swift PK, Wehausen JD, Ernest HB, Singer RS, Pauli AM, Kinde H, Rocke TE, Bleich VC (2000): Desert bighorn sheep mortality due to presumptive type C botulism in California. J Wildl Dis 36: 184–189.

Takeshi K, Fujinaga Y, Inoue K, Nakajima H, Oguma K, Ueno T, Sunagawa H, Ohyama T (1996): Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by ploymerase chain reaction. Microbiol Immunol 40: 5–11.

Trueman KF, Bock RE, Thomas RJ, Taylor JD, Green PA, Roeger HM, Ketterer PJ (1992): Suspected botulism in three intensively managed Australian cattle herds. Vet Rec 130: 398–400.

Wobeser G, Baptiste K, Clark EG, Deyo AW (1997): Type C botulism in cattle in association with a botulism die-off in waterfowl in Saskatchewan. Can Vet J 38: 782.

Woudstra C, Skarin H, Anniballi F, Fenicia L, Bano L, Drigo I, Koene M, Bayon-Auboyer MH, Buffereau JP, De Medici D, Fach P (2012): Neurotoxin gene profiling of *clostridium botulinum* types C and D native to different countries within Europe. Appl Environ Microbiol 78: 3120–3127.

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