Paper

Presence of *Clostridium botulinum* and botulinum toxin in milk and udder tissue of dairy cows with suspected botulism

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Clostridium botulinum **is an anaerobic spore-forming bacterium prevalent in the environment, and causes botulism in man and animals via toxins. Dairy cattle may be contaminated or infected by feed, water or other environmental factors. Milk may also carry the pathogen. Hence, milk and udder samples need to be tested. The number of clinical cases of bovine botulism in Germany has been increasing since the mid-1990s. Besides routine samples, additional 99 milk samples from 37 farms, and 51 udder samples from 51 farms from sick animals presumably affected by botulism were tested microbiologically by the mouse bioassay. Milk from three farms (8.1 per cent) contained botulinum toxin, and from two (5.4 per cent) bacterial states of *C botulinum*. Ten udder samples (19.6 per cent) contained toxin, and 7 (13.7 per cent) bacterial forms, including one case where both toxin and bacteria were found. The findings are discussed. Positive milk samples containing botulinum toxin or bacteria raise concern of food safety for the human consumer. Pathological udder samples may show either infection prior to, or contamination after death.**

Introduction

Botulism is a multifactorial clinical syndrome caused by different types and subtypes of *Clostridium botulinum* toxin (BoNT). The toxin may be taken up orally (intoxication) or produced inside the digestive tract (infection). Mixed forms occur (toxico-infection), but this term is not used consistently (Böhnel and Gessler 2010). BoNT may affect many physiological and regulatory networks and signalling pathways (Böhnel and Gessler 2004, 2005).

The course of the disease in cattle varies from peracute to chronic intoxication (days), or as long-lasting infection (weeks to months) with sometimes final acute outcome (Graham and Schwarze 1921, Haagsma and Laak 1978, Notermans and others 1978, AFSSA 2002, Yeruham and others 2003, ACMSF 2006b, 2009). In long-lasting toxico-infections, BoNT production occurs mainly in the distal parts of the intestinal tract ('visceral botulism'). Although not firmly established, this term is proving useful as a working model (Böhnel and others 2001, Schwagerick and Böhnel 2001). This etiology is akin to idiopathic tetanus in large animals (Mayhew 2009).

Diagnosis in sick animals is based mainly on history and clinical investigation findings, and may be supported by laboratory diagnosis, that is, proof of BoNT or toxigenic bacteria in the gastrointestinal tract (ACMSF 2006b). Clinical botulism, especially as a long-lasting infection, is suspected mainly as a herd problem due to reduced milk yield, sudden death, progressive lameness, recumbency or paralysis, dehydration, increased calf mortality and when other diseases are excluded. In mild cases, there is no clinical difference between intoxication and toxico-infection (Böhnel and others 2001, Schwagerick and Böhnel 2001). Impaired, or missing reflexes (especially eye, ear, anus), are signs of botulism in animals with otherwise unspecified symptoms (B Schwagerick, personal communication). Reduced feed and water intakes, followed by dehydration without general paralysis and sometimes recovery, have already been reported in 1921 (Graham and Schwarze), and for type B intoxications (Haagsma and Laak 1978, Notermans and others 1978, Bruckstein and Tromp 2001, Yeruham and others 2003).

Recently, Lindström and others (2010) reviewed the international literature on *C botulinum* in cattle and dairy products, and Carlin (2011) the origin of bacterial spores contaminating food.

In France, there was an increase in bovine botulism since 1990 (AFSSA 2002), and in England and Wales from 2003 onwards (Payne and others 2011). In Germany, there has been a clear rise in cases of botulism in dairy farms since the mid-1990s (Böhnel and Gessler 2003). In more than 1100 farms, clinical botulism was confirmed by laboratory tests in the years 1996–2010 (Böhnel and Gessler 2012). Hence, it seemed possible that the risk of food-borne botulism for the consumer by dairy products may also have increased. To the best of our knowledge, there are no reports on findings of *C botulinum* or BoNT in the milk of healthy cows.

There are no precise data as to when an animal has to be considered as 'sick; especially from chronic diseases, and hence, as a precaution, milk should not be delivered for human consumption. There have been proposals for a two-week restriction on delivery of milk from the whole pen even when not all animals are sick, or from healthy animals in affected farms (Cobb and others 2002). This proposition did not solve the problem of spores in the farm environment, and was subsequently withdrawn (Aish and others 2006, ACMSF 2009).

The first cases of confirmed human toxico-infectious botulism were reported from Germany. Farmers of dairy herds with visceral botulism having close contact with the animals, showed clinical motor and autonomic dysfunctions in the farms (Dressler and Saberi 2009, Krüger and others 2012, Rodloff and Kruger 2012).

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German government authorities urged investigations in this public health issue to be done to obtain information regarding the prevailing situation. Bacterial forms of *C botulinum* or BoNT may be present in cases of visceral botulism which might not be detected by actual laboratory tests (BfR 2010). To get more information on the topic 'botulism in dairy farms—health risks for the consumer' the presented study was undertaken on the basis of routine laboratory diagnostic work.

Material and methods

Attending veterinarians were asked to send, in addition to standard specimens for routine diagnosis, also milk and udder tissue samples from clinically sick or dead cows from farms suspected or confirmed as being affected by acute or chronic botulism. No information was available on clinical features or possible sources of pathogens of the particular animals. Milk samples were taken directly at the farm during milking, according to German regulations, for bacteriological milk examination. They were frozen directly after sampling. In Germany, it is not allowed to take samples for pathology at the farm. According to information of the rendering plants, udder tissue samples were taken within 24 hours after natural death or after euthanasia, and frozen thereafter. These animals had been milked just the day before death occurred. Samples were shipped frozen to the laboratory and kept at 6°C until testing.

To date, the only internationally accepted routine test for clinical or pathological specimens is the mouse bioassay with toxin neutralisation to assess the biological activity of all types of BoNT (CDC 1998, AOAC 2001). In short, toxic substances in specimens were eluted by glycerin-phosphate-buffer (pH 6.2; 1:1 w/v) overnight at 6°C. Potentially toxinogenic bacteria were cultured in clostridial medium anaerobically at 37°C for 4 days. Possible progenitor toxins were activated by adding trypsin solution. Presence of toxin was proven by intraperitoneal injection of a suspected toxic solution, culture supernatant, or milk into mice. Injection volume was 0.5 ml. Milk was used directly (0.8 ml). The animals were kept under close supervision for 96 hours for typical signs of illness or death. If there were signs indicating the presence of toxic substances, toxin neutralisation was carried out using type-specific botulinum antitoxins. The initially lethal sample was mixed with antitoxin and kept for 30 minutes at 37°C before injection. To save animals, in our laboratory, monospecific antitoxin types A, B and E were pooled, as were C and D. If specific neutralisation of the toxin led to the survival of the mice, this result was considered as being conclusive. Results are marked for these two groups as ABE or CD. Neutralisation of a single sample with both ABE and CD or even ABECD together, was considered inconclusive and marked as negative (Gessler and Böhnel 2004). If one particular animal was tested positive, the diagnosis of botulism was applied for the whole farm.

The seasonal entry of samples and the results were compared by Fisher exact test.

The actual laboratory findings were compared with the health status including the results of the routine testing of the originating farm. The standard samples for suspected botulism are contents of rumen and caecum, and blood serum of living animals. Additionally, content of jejunum and liver specimens were sent for autopsies. Bovine botulism is not a notifiable disease in Germany, hence, there are no legal case definitions of the disease. Clear clinical features are a decisive means of diagnosis if other diseases like, for example, milk fever or rabies are excluded (AFSSA 2002, ACMSF 2006b, 2009). According to the human case definition (eg, CDC 2011), detection of toxin in clinical specimens of animals with clinical signs of the disease is considered here as a definitive criterion, and detection of *C botulinum* in faeces of an animal with clinical features is strongly suggestive.

Results

From 2002 to 2010 in Germany, 99 milk samples from 37 farms, and 52 udder specimens from 52 farms affected by botulism were received and tested accordingly. One udder sample arrived decomposed after a misdirected transport over several days. Thus, 51 udder samples were listed. Most of these samples were included in shipments of routine samples. In some cases, no additional samples were available for bacteriological examination. There were no instances of both milk and udder samples from the same farm.

The results of the laboratory tests are presented in Table 1 for milk samples, and in Table 2 for udder specimens, and compared with bacteriological results of routine diagnostic specimens where detailed laboratory results are not given. Five farms out of 37 (13.5 per cent) had positive milk samples (*C botulinum* or BoNT), and 17 from 51 (33.3 per cent) positive udder samples. BoNT was found in three milk samples and 10 udder samples; bacteria were found in two milk samples and in seven udder specimens, including one udder sample, in which both BoNT and bacteria were detected. There was no proof of progenitor toxins. Trypsinised samples showed no specific results. There were no positive milk or udder samples from farms without confirmed botulism status. For one udder sample, no information on the farm health status was available. The botulinum types in milk and udder tissue correspond with those of the farm health status except in one udder sample.

The seasonal distribution of the samples is given in Fig 1, indicating positive results for *C botulinum* or BoNT. A statistical analysis of the udder samples was carried out using the Fisher exact test. There was no likelihood of the season having an impact on the number of positive laboratory results (significance level α=0.05).

Discussion

C botulinum and its toxins in dairy products may be technically difficult to trace by using the actual laboratory tests available for routine testing (Lindström and Korkeala 2006, Lindström and others 2010). Spores may be concentrated by centrifugation of milk to improve the sensitivity of test methods (Ágoston and others 2009).

Even in positive clinical cases, no more than 50 per cent of all laboratory tests provide an affirmative result (Popoff 1989, Gessler and

TABLE 1: Results of microbiological examination of milk samples compared with farm health status (detailed results of routine samples not shown) from clinically affected farms

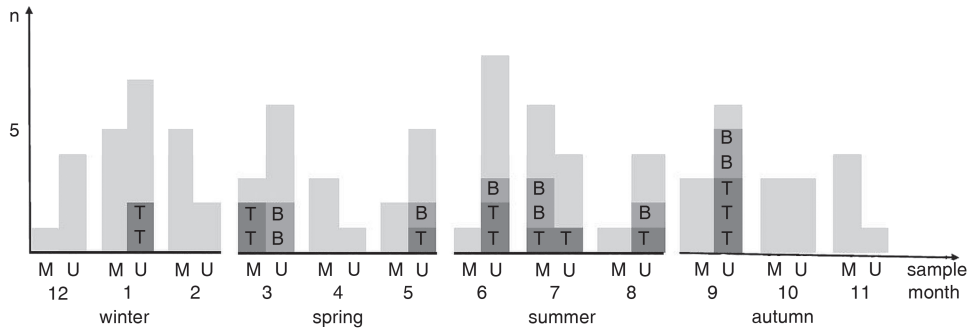
		Laboratory diagnosis		
		Milk samples		
Farms (n)		Botulinum toxin	*Clostridium botulinum*	Routine samples
	7	Neg.	Neg.	Neg.
	4	Neg.	Neg.	n.t.
	6	Neg.	Neg.	ABE
	2	Neg.	Neg.	ABE+CD
	13	Neg.	Neg.	CD
	2	ABE	Neg.	ABE
	1	CD	Neg.	ABE+CD
	1	Neg.	ABE	Neg.
	1	Neg.	ABE	ABE+CD
Total	37	3	2	25

ABE and CD, Grouping of *C botulinum* serotypes; Neg., No laboratory proof of *C botulinum* or botulinum toxin; n.t., Clinically suspect, no routine samples tested.

TABLE 2: Results of microbiological examination of udder tissue samples compared with farm health status (detailed results of routine not shown) from clinically affected farms

		Laboratory diagnosis		
		Udder samples		
Farms (n)		Botulinum toxin	*Clostridium Botulinum*	Routine samples
	10	Neg.	Neg.	neg.
	4	Neg.	Neg.	ABE
	1	Neg.	Neg.	ABE+CD
	20	Neg.	Neg.	CD
	2	ABE	Neg.	n.t.
	1	ABE	Neg.	CD
	6	CD	Neg.	CD
	1	Neg.	ABE	n.t.
	2	Neg.	ABE	ABE
	3	Neg.	CD	CD
	1*	CD	CD	CD
Total	51	10	7	38

ABE and CD, Grouping of *C botulinum* serotypes; Neg., No proof of *C botulinum* or botulinum toxin; n.t., Clinically suspect, no routine samples tested.
*Toxin + bacteria in the same sample



Light grey squares = number of negative cased;medium grey squares B = positive for *C. botulinum;* dark grey squares T=positive for botulinum toxin; M=milk; U=udder

FIG 1: Seasonal distribution of samples. Light grey squares, number of negative cases; medium grey squares B, positive for Clostridium botulinum; dark grey squares T, positive for botulinum toxin; M, milk; U, udder

Böhnel 2004, Lindström and Korkeala 2006, Cai and others 2007, Lindström and others 2010). Only positive results are considered being confirmatory. Negative ones may be due to lacking in sensitivity.

The presence of BoNT in three (2×ABE, 1x×CD), and *C botulinum* (ABE) in two milk samples, raise direct concerns on food safety, as types A, B and E are considered more dangerous for human beings (CDC 1998). The small number of tested samples here, and the uncertainties of the clinical history of the animals, do not allow making a risk assessment by the presented results.

The general opinion is that milk of sick (intoxicated) cows does not contain BoNT as no transfer takes place from blood to milk (Smith and Sugyiama 1988, Galey and others 2000, without indication of toxin type Moeller and others 2003, 2009 for type C). However, Graham and others (1922) showed a health risk from the milk of cows and sows fed on infected feed (type A). Teat infection was demonstrated, and toxin formation within an infected udder was presumed. Moberg and Sugiyama (1980) found toxin type A in the milk of artificially intestinally infected rats. In 2002, we found BoNT type B in the milk of a cow which suffered from visceral botulism, as well as from mastitis on the day when it was sent for slaughter (Böhnel and others 2004). The pathogen may enter the udder via the teat canal, followed by bacterial multiplication and toxin production. This may be only an infection which need not lead to mastitis. There are no reports on the effect of long-lasting non-detectable amounts of toxins in serum. As was shown, toxin may be present in the buccal cavity. A resorption may take place without the stomach passage (Böhnel and others 2008). The stability of type B and C toxins in ruminal contents was investigated by Kozaki and Notermans in 1980. Finding of bacterial states or spores, together with compatible clinical signs, support the presumptive diagnosis of botulism. Toxin types A, B, E are considered being more important for human beings than types C and D which are apparently less pathogenic (Smith and Sugiyama 1988). Types C and D, and especially their mosaic forms C/D and D/C, are highly toxic for animals (Nakamura and others 2010, Woudstra and others 2012).

BoNT is believed to be destroyed during milk pasteurisation. However Rasooly and Do (2010) reported heat stability for toxin type B. In raw milk products, enzymes may reduce or destroy its toxicity. Vegetative forms of *C botulinum* are destroyed by ordinary hygienisation processes. Spores may survive even higher temperatures (Julien and others 2008) and may lead to toxin production, either in the product or following ingestion, in the intestinal tract (Bell and Kyriakides 2000, Lindström and others 2010).

Although *C botulinum* is not invasive (Smith and Sugiyama 1988), neither the possibility of an udder infection prior to death, nor an invasion of the bacteria after death via teat channel or blood vessel could be ruled out. The origin of *C botulinum* and BoNT in the udder samples remain unknown. There is no statistically relevant seasonal difference in the number of positive laboratory results. The outside temperature during transport of the carcases to the rendering stations seems to have no influence on bacterial proliferation or toxin production.

Delivery of milk from sick animals is not allowed under German legislation. Sick and convalescent animals, as well as apparently healthy animals, may excrete the pathogen *C botulinum* with faeces or saliva, thus risking creation of a vicious circle by increasing the numbers of spores in the environment (Notermans and others 1981, AFSSA 2002, Böhnel and others 2008, Julien and others 2008, Böhnel and Gessler 2010). Toxico-infections occur and persist even after acute non-lethal intoxications (type B) (Haagsma and Laak 1978, Notermans and others 1978). The environment and the type of bacteria influence sporulation (Carlin 2011). A bioterrorist background for the presence of *C botulinum* or its toxins is not considered here (Wein and Liu 2005, Weingart and others 2010). Feed management may improve the health status of animals and reduce the risk of milk contamination (Graham and others 1922, Kalač 2011).

As there have been numerous clinical outbreaks of bovine botulism in recent years, an improved risk analysis should be set up for dairy production (Augustin 2011), and an actual risk assessment has to be reconsidered (Popoff and Argente 1996, AFSSA 2002, ACMFS 2006a, b, 2009). Unknown transport and storage conditions of dairy products by the consumer or any accidental and deliberate microbiological contamination in the feed and food chains should be included (Knutsson and others 2011, Malakar and others 2011). It should be emphasised that the pathogen may spread, mainly as spores, by aerosols or surface contamination within different areas of a dairy, where a variety of milk products intended for addition to novel food preparations are being stored (Carlin 2011).

In 2000, Bell and Kyriakides suggested a practical approach for controlling *C botulinum* in food. The presented results have important implications on reconsidering the whole complex of the zoonosis 'botulism' (EU 2003, EFSA 2005). International reference preparations for botulinum laboratory diagnosis should be made available. An interlaboratory comparison and international research project in this field would be useful to confirm positive results, as the implications are potentially rather serious. Statistically relevant testing of healthy and sick cows from healthy and affected farms should be undertaken. Payne and others (2011) suggest the area of possible enteric and periparturient botulism-associated syndromes as a useful area for future study. In further investigations, the general clinical feature, and especially the possible role of mastitis parameters in toxicogenesis, should be considered. Emphasis should be laid on progenitor toxins and toxins of all different *C botulinum* types and other clostridia-producing BoNT, as the actual mouse bioassay covers only biologically active toxins in the mouse. Modern molecular biological testing methods may help to test the necessary large number of samples, and to reduce the use of test animals for ethical reasons.

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Competing interests None.

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