

Equine botulism and acute pasture myodystrophy: New soil-borne emerging diseases in Switzerland?

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Summary

In Switzerland, the incidence of equine botulism and acute pasture myodystrophy have remarkably increased in the last five years. Equine fodder-borne botulism in Europe is most likely caused by *Clostridium botulinum* types C and D that produce the toxins BoNT/C and BoNT/D. Horses showing signs suggestive of botulism (muscle weakness and tremors, reduced tongue tone, slow chewing, salivation and difficulties swallowing, drooping eyelids, mydriasis), especially patients that have fed on suspect fodder (mostly haylage), must be treated with anti-serum as soon as possible. They also need intensive care, which is often difficult to provide and always expensive in the face of a guarded to poor prognosis. Therefore, prevention (high standards of forage quality and vaccination) is all the more important. Pasture myodystrophy is an acute disease with signs of rhabdomyolysis and lethality rate over 90%. It affects grazing horses under frosty, windy and rainy conditions. Preliminary results indicate that *Clostridium sordellii* and *Clostridium bifermentans* producing lethal toxin may play a role in pasture myodystrophy. Our efforts concentrate on developing a new subunit vaccine for equine botulism and understanding the ethiology and pathogenesis of pasture myodystrophy with the goal of improving prevention against these highly fatal diseases that present a significant risk to our horse population.

Keywords: horse, rhabdomyolysis, paralysis, clostridia, toxin

Botulismus und Weidemyopathie beim Pferd: Verursachen bodenbewohnende Keime neue Krankheiten in der Schweiz?

Fälle von Botulismus und Weidemyopathie haben in der Schweiz während der letzten 5 Jahre deutlich zugenommen. Durch Futter verursachter Botulismus ist in Europa höchstwahrscheinlich auf *Clostridium botulinum* Typen zurückzuführen, die BoNT/C und BoNT/D Toxine produzieren. Botulismusverdachtsfälle – Pferde, die Muskelschwäche und -zittern, verminderten Zungentonus, langsames Kauen, Speicheln, Schluckstörungen, sowie Ptosis und Mydriasis zeigen – müssen ohne Verzug mit Antiserum behandelt werden, v.a. wenn sie verdächtiges Futter, meist Haylage/Grassilage, gefressen haben. Solche Patienten bedürfen darüber hinaus einer intensiven Pflege, die manchmal schwierig zu gewährleisten ist. Ausserdem ist diese intensive Therapie teuer, und die Prognose trotzdem ungünstig bis schlecht. Umso wichtiger ist darum die Prophylaxe: allgemein ist auf beste Futterqualität zu achten und Tiere in Risikobeständen sollten geimpft werden. Die Weidemyopathie, eine akute Rhabdomyolyse mit einer Letalität von über 90%, tritt v.a. bei Pferden mit Weidegang bei kalten, windigen und regnerischen Bedingungen auf. Vorläufige Resultate zeigen, dass lethaltoxin-produzierende *Clostridium sordellii* und *Clostridium bifermentans* bei der Weidemyopathie eine Rolle spielen könnten. Wir konzentrieren unsere Anstrengungen auf die Produktion einer neuartigen Botulismus – «Subunit» – Vakzine und auf die weitere Erforschung der Ätiologie und Pathogenese der Weidemyopathie. Unser Ziel ist es, die Prävention dieser hochlethalen Krankheiten, die ein ernsthaftes Risiko für unsere Pferde darstellen, zu verbessern.

Schlüsselwörter: Pferd, Rhabdomyolyse, Paralyse, Clostridien, Toxin

Introduction

In Switzerland, the incidence of equine botulism and acute pasture myodystrophy have remarkably increased in the last five years. In totally over 60 000 horses treated at the equine clinic of the Vetsuisse Faculty of the University of Bern between 1960 and 2000, neither disease has been clinically diagnosed, bacteriologically nor toxicologically confirmed and, to the best of our knowledge, no description of cases in Switzerland can be found in the literature of the last century. Since November of 2001, 38 horses have developed symptoms highly suggestive of botulism. All of these cases occurred in groups of horses fed haylage, 30 of them were euthanised. In the same time period, 26 cases with symptoms of pasture myopathy were recorded, of these 19 died. Outbreaks of both diseases that were reported to us were predominantly localised to the West and North-West of Switzerland, a region that has a high equine population density. Due to the emergence of these highly lethal diseases in our horse population, we are reviewing the clinically relevant presentation of botulism and pasture myodystrophy, and we present the rationale for our efforts to develop an improved botulism vaccine. Furthermore, we hypothesise that the well-known pathogenesis of botulism may offer clues to the as yet completely mysterious aetiology of pasture myodystrophy.

Botulism in horses

Botulism is a paralytic disease caused by the neurotoxins of *Clostridium (C.) botulinum*, which can be found in soil samples throughout the world. With a lethal dose to humans of less than 1 mcg, botulinum neurotoxin is the most poisonous substance known. *C. botulinum* that produces the neurotoxins is a Gram-positive, obligatory anaerobic, rod-shaped, endospore-forming bacterium found in soil, sewage, marine sediments and in the intestinal tract of many animals, particularly of aquatic birds and poultry. Spores are metabolically dormant and resistant to heat up to 120 °C, radiation, desiccation, extreme pH values and disinfectants. Germination of *C. botulinum* is triggered under anaerobic conditions, pH > 4, and by host or pathogen specific nutrients called “germinants”, such as amino-acids sugars, ribosides and certain ions (Böhnel and Gessler 1999; Duchesnes and Mainil 2002; Duchesnes et al. 2004). Immediately after germination, production of the botulinum neurotoxin (BoNT) starts. There are seven types of *C. botulinum* designated *C. botulinum* type A through G producing each a specific botulinum toxin BoNT/A through G. The various botulinum toxins all cause the same symptoms caused by paresis and paralysis, although

their molecular mechanisms of actions at the neuromuscular junction as well as their receptor specificities are different. The various types of *C. botulinum* are known to show species specificity, particular geographical prevalence as well as type-specific occurrence in various sources of intoxication.

Equine botulism in America is predominantly caused by *C. botulinum* types A and B that secrete the toxins BoNT/A and BoNT/B, respectively. In Europe, it is most likely caused by types C and D that produce the toxins BoNT/C and BoNT/D (e.g. Dietz, 1998). However, an outbreak in horses due to an unclassified botulinum toxin has also been reported in Germany (Wollanke, 2004). Botulinum toxins can also be produced by neurotoxicogenic strains of *C. butyricum* and *C. baratii* (Schiavo et al., 2000; Duchesnes et al., 2004). There are three main forms of botulism. Fodder-borne botulism, the most common form in horses, is caused by uptake of toxin contaminated fodder or water. The toxico-infectious form, the so called “shaker foal syndrome”, occurs in foals after ingestion of spores, which germinate in the intestines and release toxin. Contamination of deep wounds can lead to the very rare form of wound botulism. All forms of botulism can be fatal and are considered emergencies. In the following, however, we concentrate on clinically relevant characteristics of fodder-borne botulism, the only form of botulism in horses observed in Switzerland to date. The severity depends on the amount of toxin ingested. However, since horses as a species are particularly sensitive to botulinum toxin, ingestion of only a small hand-full of contaminated fodder can be enough to produce clinical disease.

Clinical signs, diagnosis, prognosis and treatment

The classic symptoms of botulism in *equidae* include general muscle weakness and tremors (therefore the term “shaker foal”), reduced tongue tone, slow chewing, dripping of saliva and dropping of food, drooping eyelids, mydriasis and difficulties swallowing (e.g. Dietz, 1998; Wollanke, 2004). Importantly, although the patients may be somewhat depressed, there are no signs of central nervous system involvement: Symptoms generally begin 18 to 36 hours post intoxication, but they can occur as early as 6 hours or as late as 10 days after eating contaminated food. When untreated, these symptoms progress to cause paralysis of the legs, trunk and, finally, respiratory muscles. Lethality rate in recumbent animals is almost 100%. Even animals that can stay in sternal recumbency or remain standing have a poor prognosis. In animals that survive, recovery takes about 3 weeks, which is the time necessary for remodelling of motor end-plates (Popoff, 1996; Böhnel and Gessler, 1999; Popoff, 2003; Duchesnes et al., 2004).

A tentative diagnosis is based on history and physical examination. However, the above-described signs are not specific, especially at the onset of the disease. Other intoxications and neuropathies, particularly Grass Sickness and Equine Motor Neuron Disease, may show similar signs (Hunter and Poxton, 2001). In the course of the disease, the clinical picture becomes more specific, but treatment with anti-serum has no effect on toxin that is already bound to the nerve. The mouse protection assay is the official European diagnostic test. Since horses are much more susceptible to BoNT/C and BoNT/D than mice, equine botulism caused by *C. botulinum* type C and D is very difficult to confirm in the laboratory and false negative laboratory diagnosis is the rule rather than the exception. None of the cases diagnosed clinically in Switzerland could be confirmed by testing serum. Testing stomach contents from acutely affected horses is a possibility, but great care must be taken when siphoning by stomach tube from a suspected case- no aspiration by mouth! Whenever possible, suspect fodder should be cultivated and subsequently tested in the mouse assay for the presence of botulism bacteria and toxin. When an animal dies, cultivation from ingesta and liver and subsequent testing is currently the only way to confirm a diagnosis of botulism.

Therefore, it is imperative that horses showing signs suggestive of botulism, especially patients with a history of feeding on suspect fodder (see below), be treated with anti-serum as soon as possible. Of course, other animals that have also been in contact with the suspect source should be treated as well. All other therapeutic measures are symptomatic, but none the less important: administration of water, electrolytes and energy by naso-gastric tube or parenterally to dysphagic animals (Gerber et al., 2006), sternal support or slinging of recumbent patients as well as respiratory support (O₂; respirator in foals) and antibiotic prevention of pneumonia. In cases of wound-botulism and toxico-infectious botulism antibiotic (penicillin) administration is necessary in addition to antitoxin. Such intensive care is often difficult to provide for adult horses and always expensive in the face of a guarded to poor prognosis. Therefore, prevention, which is based on assuring high standards of forage quality and vaccinating horses at risk, is all the more important.

Prevention – forage quality

As outlined above, equine botulism in Switzerland has emerged with the increasing use of haylage (grass silage). Good quality haylage is an ideal source of roughage for horses due to its nutritional value, digestibility, palatability as well as ease of harvesting

(less weather dependent than hay) and of storage. The near absence of inhalable allergens and irritants makes it ideal for horses with lower airway disease. Important to note, however, that we found a benefit of haylage over hay only for patients with clinical signs like coughing and respiratory distress, but not for horses that simply have subclinical airway disease (Boog et al., 2005). In any case when feeding silage to horses, highest standards of quality must be assured by avoiding the use of poultry litter as fertilizer on pastures, soil contamination in haylage or silage (the grass should not be cut shorter than 10 cm from the ground), pH > 4.5 during fermentation, dry-matter content of less than 25 or more than 45 percent, production of haylage or silage from meadows that are densely populated by small rodents and birds, and inadequate storage (breakage of plastic seal, mechanically or by rodents).

Prevention – vaccination

The goal of protective vaccination against botulism is to generate neutralizing antibodies against botulinum neurotoxins. Commercial botulinum vaccines are formalin-inactivated culture supernatants from *C. botulinum* grown in a fermentor. Such vaccines are efficient, their production, however, is very expensive and time consuming due to drastic safety measures that must be observed in the production process. Furthermore, current vaccines against botulism cause strong side reactions due to the harsh chemical substances used in the inactivation processes of the toxins. Worldwide, only one vaccine against *C. botulinum* type C and D (Botulism Vaccine®; Onderstepoort Biological Products, RSA) and one against *C. botulinum* type A and B (Bot Tox-B®; Neogen Corporation, USA) are licensed for use in horses and cattle. Importantly, there is a lack of cross-immunity between antibodies against specific toxin types. To reduce vaccination side effects and to improve safety, subunit vaccines based on immunogenic non-toxic fragments of the neurotoxin are currently investigated.

All *C. botulinum* neurotoxins have a similar structure. They are synthesized in the bacterium as a single chain pre-toxin and subsequently proteolytically cleaved into a light chain of approximately 50 kDa (L-chain) and a heavy chain of approximately 100 kDa (H-chain) which are held together by a disulfide-bridge (Schiavo et al., 2000). A Zn-dependent metalloprotease confers toxic activity to the L-chain. The H-chain enables specific recognition of the receptor and translocation of the toxic L-chain into the neuron. It has two major domains, both necessary to interrupt normal neurotransmitter release from the nerve terminus to the muscle cell (Fig. 1A). The C-terminal half of the H-chain, fragment Hc,

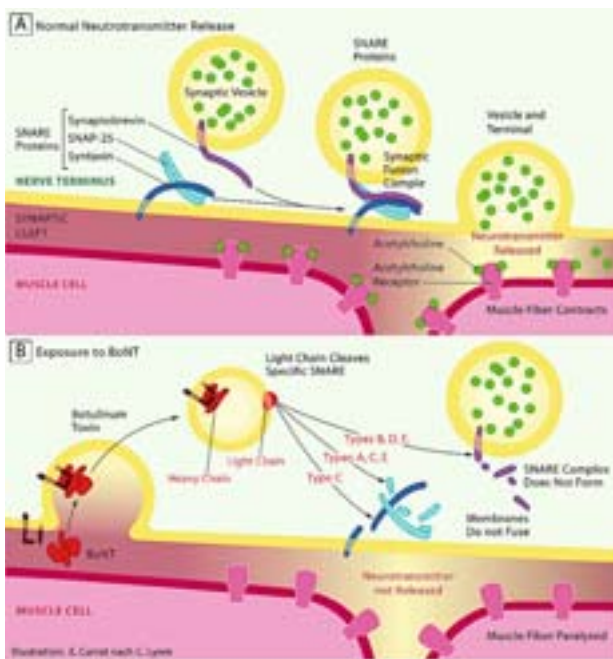


Figure 1, A: Normal neurotransmitter release from the nerve terminus to the muscle cell illustrating specifically the roles of SNAP 25, Syntaxin or Synaptobrevin, which are the three SNARE (soluble N-ethylmaleinamid-sensitive factor attachment protein receptor) proteins that form the synaptic fusion complex. B: Attachment of the heavy chain to a surface receptor, which mediates the endocytosis into the cytoplasm of the neuron, and the type-specific protease-activity of the toxic L-chain cleaving the SNARE proteins, which prevents membrane fusion and neurotransmitter release.

recognizes a surface receptor on neurons, which provokes the internalization of the whole toxin into the endocytic vesicles (Tsukamoto et al., 2005). The N-terminal half of the H-chain mediates the translocation of the toxic L-chain, into the cytoplasm of the neuron (Fig. 1B). In the motor nerve endings and autonomic cholinergic junctions, the L-chain by its protease activity cleaves SNAP 25, Syntaxin or Synaptobrevin, which are the three SNARE (soluble N-ethylmaleinamid-sensitive factor attachment protein receptor) proteins that form the synaptic fusion complex that has a determinant role in neuroexocytosis. As illustrated in Figure B, BoNT show specificity: e.g. BoNT/C is specific to SNAP 25 and Syntaxin while BoNT/D cleaves Synaptobrevin (Schiavo et al., 2000; Breidenbach and Brunger, 2005). Finally, all BoNT block the Ca^{2+} induced release of acetylcholine and causes flaccid paralysis.

A successful strategy to develop a new vaccine against *C. botulinum* type A has been proposed on the basis of recombinant Hc fragment of BoNT/A (Clayton et al., 1995; Byrne and Smith, 2000). The group of M. Popoff at the Pasteur Institute in Paris showed that the Hc fragment of the heavy chain of BoNT/A, containing the 459 C'-terminally located amino-acids is essential for the generation of protective

antibodies against botulinum toxin type A (Tavallaie et al., 2004).

Our current research efforts to improve prophylaxis of equine botulism focuses on the possibility to induce toxin neutralizing antibodies by immunizing horses with recombinant, purified proteins of the Hc fragment of BoNT/C and BoNT/D. Preliminary results of a study performed with the Hc fragment of BoNT/C reveal that the antigen is able to induce neutralizing antibodies in horses as indicated by the mouse protection assay with serum from vaccinated horses. Furthermore, testing for adverse reactions with vaccines prepared from recombinant Hc fragment proteins show a significantly better clinical tolerance (specifically; less swelling at injection sites) in vaccinated horses compared to the classical vaccines currently on the market (Eberle, Straub, Frey; unpublished results). An efficient vaccine against equine botulism in Europe, however, will require the development of a bivalent vaccine containing Hc fragments from both BoNT/C and BoNT/D. This approach might also be beneficial for the protection of horses from Grass Sickness, another disease that may be linked to *Clostridium botulinum*. Hunter and Poxton (2001) suggest that high antibody titers against BoNT/C may be protective against Grass Sickness. Interestingly, other groups have hypothesised that amyotrophic lateral sclerosis, a human disease that shares many similarities with EMND, may also be caused by clostridial neurotoxins (Longstreth et al., 2005). These findings, complemented by our observations on the epidemiology of pasture myodystrophy, which suggest a soil-borne agent, gave rise to our hypothesis that pasture myodystrophy may also be caused by neurotoxin producing *Clostridium* species, the target of the presumed toxin being the myocyte.

Pasture myodystrophy

Pasture myodystrophy, also called atypical myopathy or atypical myoglobinuria, is a fatal disease with a lethality rate over 90% that affects grazing horses while at pasture under frosty, windy and rainy conditions. It affects horses of any age, breed or sex. The disease has been described over 60 years ago, but its etiological agent still remains elusive, despite the investigation of potential causative agents such as herbicides, plant or fungal toxins (Whitwell et al., 1988; Votion et al., 2003; Gehlen et al., 2005). Details of outbreaks of the disease in England, Germany and in Belgium are well documented in the literature (Hosie et al., 1986; Whitwell et al., 1988; Brandt et al., 1997; Delguste et al. 2002). In the last four years over 100 cases of pasture myodystrophy in France, Belgium, Germany as well as 26 cases in Switzerland were diagnosed.

Clinical signs, diagnosis, treatment and pathological findings

The acutely affected horses present a sudden onset of stiffness and muscular weakness and tremors. Most cases progress rapidly to lateral recumbency, which in some cases is the initial sign. Most horses die within 48 hours. Horses that survive show various degrees of muscle wastage of which they can fully recover. Proper diagnosis can be achieved by measuring the enzyme activity of creatine phosphokinase (CPK) or by analysis of urines for presence of myoglobin. CPK activities above 20000 I. E. indicate a rather poor prognosis. Horses should be treated like exertional rhabdomyolysis patients. Supportive therapy should be given in form of analgesics, intravenous fluids with micromolecular plasmaexpanders, vitamin E and Selenium preparations. Gross pathology may show gross muscle lesions like intramural myocardial and diaphragmatic haemorrhages. Histopathology shows distinct acute rhabdomyolysis. The observation that type I muscle fibres seem to be more susceptible to the pathogen than other fibres (Brandt et al., 1997), could not be confirmed in our cases.

Clostridial toxico-infections as a possible cause of pasture myodystrophy

To test our hypothesis that this highly fatal disease is caused by clostridia, we investigated ingesta and faeces samples of affected horses for the presence of *Clostridium* species. Preliminary results indicate that *Clostridium sordellii* and *Clostridium bifermentans* producing lethal toxin (LT) may play a role in pasture myodystrophy (Straub, Frey; unpublished results). *Clostridium sordellii* infections often result in toxic shock and multi-organ failure in human and also various animal species, due to toxin induced necrosis of various cell types.

LT of *C. sordellii*, a 250-kDa protein, is the bacteria's major virulence factor and belongs to a family of large

clostridial cytotoxins. The mechanism by which LT elicits necrosis of skeletal muscle fibres has not been completely elucidated, but it appears to be related to its ability to glucosylate small guanosin triphosphate (GTP)-binding proteins. The study of Barbier et al. (2004) demonstrates that the intramuscular injection of a single sublethal dose of *C. sordellii* LT causes significant damage to skeletal muscle fibres in mice. Also, it is likely that the toxin has an effect on endothelial cells located at the interface between blood and skeletal muscle, leading to the expression of proinflammatory mediators, vascular leakage, and subsequent ischemia of skeletal muscle tissue. Indeed, *C. sordellii* LT has been shown to disrupt the actin microfilament system and the monolayer integrity of primary cultured human endothelial cells (Vouret-Craviari et al., 1999). These results support our hypothesis that *C. sordellii* and *C. bifermentans* are contributory or causative factors in the pathogenesis of acute myodystrophy of grazing horses. Current investigations focus on the epidemiology of toxigenic *C. sordellii* and *C. bifermentans* and on the role of large clostridial toxins in pasture myodystrophy, which are essential to develop preventive strategies against this disease.

In summary, our observations suggest that equine botulism and acute pasture myodystrophy are emerging soil-borne diseases in Switzerland. Our efforts concentrate on developing a subunit vaccine with less side effects for the former and understanding the ethiology and pathogenesis of the latter with the goal of improving prevention against both these highly fatal diseases that present a significant risk to our horse population.

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Botulisme et myopathie atypique chez le cheval: Des germes telluriques causent-ils de nouvelles maladies en Suisse?

Les cas de botulisme et de myopathie atypique ont nettement augmenté en Suisse ces 5 dernières années. Le botulisme causé par l'alimentation est, en Europe, très vraisemblablement à mettre sur le compte de divers types de *Clostridium botulinum* qui produisent des toxiques BoNT/C et BoNT/D. Les chevaux qui présentent une suspicion de botulisme – faiblesse et tremblements musculaires, tonus lingual diminué, mastication ralentie, salivation, trouble de la déglutition, ptose, mydriase – doivent être traités rapidement avec un anti-sérum, particulièrement s'ils ont consommés des fourrages suspects de type Haylage ou ensilage d'herbe. De tels patients nécessitent en outre des soins intensifs qui sont parfois difficiles à assurer. Ce traitement est coûteux et le pronostic reste douteux à mauvais. La prophylaxie est donc particulièrement importante: il faut prendre garde à la meilleure qualité possible des aliments et on devrait vacciner les animaux stationnés sur des exploitations à risque. La myopathie atypique, une rhabdomyolyse aiguë avec une létalité de plus de 90%, touche particulièrement les chevaux mis au prés par temps froid, venteux et pluvieux. Des résultats intermédiaires montrent que *Clostridium sordellii* et *Clostridium bifermentans*, qui produisent des toxines létales, pourraient jouer un rôle dans la myopathie atypique. Nous concentrons nos efforts sur la production d'un nouveau vaccin à subunité contre le botulisme et à la recherche de l'étiologie et de la pathogenèse de la myopathie atypique. Notre but est d'améliorer la prévention de ces maladies à forte létalité qui représentent un risque sérieux pour nos chevaux.

Botulismo e miopatia atipica nel cavallo: Dei germi della terra provocano nuove malattie in Svizzera?

Casi di botulismo e di miopatia atipica sono negli ultimi 5 anni nettamente aumentati in Svizzera. La fonte del botulismo provocato dall'alimentazione, in Europa, è molto probabilmente riconducibile a diversi tipi di *Clostridium botulinum* che produce tossine BoNT/C e BoNT/D. Cavalli, che mostrano segni tipici di botulismo quali debolezza e tremito muscolare, tono linguale diminuito, masticazione lenta, salivazione, disturbi della deglutizione e ptosi e midriasi, devono essere trattati senza indugio con un antisiero, in particolare se si sono nutriti con foraggi sospetti di tipo haylage o d'erba in silage. Questi pazienti abbisognano inoltre di cure intensive che certe volte sono difficilmente eseguibili. Inoltre questa terapia intensiva è costosa e la prognosi risulta da dubitativa a cattiva. Perciò è estremamente importante la profilassi: fare molta più attenzione alla migliore qualità degli alimenti e vaccinare gli animali che si trovano in zone a rischio. La miopatia atipica è una forma acuta di rhabdomyolisi con una letalità superiore al 90% che concerne in particolare i cavalli che pascolano durante periodi freddi, ventosi e piovigginosi. Risultati intermedi mostrano che il *Clostridium sordellii* e il *Clostridium bifermentans*, che producono delle tossine letali, avrebbero un ruolo nella miopatia atipica. I nostri sforzi si sono concentrati sulla produzione di un nuovo vaccino a subunità contro il botulismo e alla ricerca dell'etiologia e della patogenesi della miopatia atipica. Il nostro scopo finale è il miglioramento della prevenzione di queste malattie altamente letali che rappresentano un serio rischio per i nostri cavalli.

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