**UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE**

**and**

**THE UNIVERSITY OF NORDLAND**

F-723/14

UNIVERSITY OF NORDLAND


# BOTULISM

**BACHELOR THESIS**

**2014**

**Johanne Sørby**

**UNIVERSITY OF VETERINARY MEDICINE**
**AND PHARMACY IN KOŠICE**

**and**

**THE UNIVERSITY OF NORDLAND**

# BOTULISM

**BACHELOR THESIS**

Study programme: Joint Study Programme – Bachelor in Animal Science
Study branch: 4320 General veterinary medicine – Animal Science
Place of studies (Department/Institute): Department of Epizootology and Parasitology
Tutor: Assoc. prof. Anna Ondrejková, D.V.M., PhD

**Košice 2014**

**Johanne Sørby**

## Concluding work assignment

| | |
|---|---|
| Name and surname, student`s titles: | Johanne Sørby |
| Title of the concluding work: | Botulism |
| Type of the concluding work : | Bachelor thesis |
| Name, surname and titles of the tutor: | Assoc. prof. Anna Ondrejková, D.V.M., PhD |
| Place of the studies ( Department/Institute ): | Department of Epizootology and Parasitology |
| Name, surname and titles of the head of the Department/Institute: | prof. Valéria Letková, DVM., PhD. |
| Annotation of the concluding work: | This thesis deals with the occurence, epizootiology and epidemiology, pathogenesis, clinical signs, diagnosis and prevention of botulism in animal and human populations |
| Language of the work: | English |
| Date of approval of the assignment: | October 16, 2013 |

..... .....

Signature of the tutor Signature of the student

## Analytical list

| | |
|---|---|
| Author: | Johanne Sørby |
| Work title: | Botulism |
| Language of the work | English |
| Type of the work: | Bachelor thesis |
| Number of pages: | 53 |
| Academic title: | Bachelor of Animal Science |
| University: | University of Veterinary Medicine and Pharmacy in Košice and the University of Nordland |
| Department: | Department of Epizootology and Parasitology |
| Study branch: | 4320 General veterinary medicine – Animal Science |
| Study programme: | Joint Study Programme – Bachelor in Animal Science |
| City: | Košice |
| Tutor: | Assoc. prof. Anna Ondrejková, D.V.M., PhD |
| Submitted on: | April 2, 2014 |
| Date of defense: | June 5, 2014 |
| Key words: | *Botulism, Clostridium botulinum, botulinum neurotoxin* |
| Conspectus category: | Agriculture; Veterinary medicine |
| Work citations: | Johanne Sørby: Botulism.<br>Bachelor thesis, Košice: University of Veterinary Medicine in Košice and the University of Nordland, 2014. 53 pp. |
| Work title in English: | Botulism |
| Key words in English: | *Botulism, Clostridium botulinum, botulinum neurotoxin* |

## Thank you note

I would like to thank my tutor, Assoc. prof. Anna Ondrejková, D.V.M., PhD. for the help with the thesis. Her knowledge was invaluable in helping outline the path I should follow, when I was undecided. Without her guidance and insight this thesis would never have been possible.

I would also like to thank Prof. Per Einar Granum from the Norwegian University of Life Sciences and Scientist Magne Kaldhusdal from the Norwegian Veterinary Institute, for providing me access to academic papers relevant to this thesis.

## Abstract

Botulizmus patrí medzi toxické infekcie; je to zvyčajne alimentárna infekčná choroba, ktorá je spoločná pre ľudí a mnohé druhy zvierat. Ochorenie je spôsobené konzumáciou potravín, resp. krmív, ktoré boli kontaminované botulotoxínom alebo obsahovali spóry *Clostridium botulinum*. Výskyt choroby je vždy považovaný za závažné ochorenie vyžadujúce lekársku pohotovosť. Rýchle rozpoznanie klinických príznakov, zistenie možného prameňa nákazy, resp. kontaminácie potravy sú kľúčové pre zabránenie vzniku ďalších prípadov infekcie a včasného podania antitoxínu. Bez vykonania rýchlej liečby, dochádza k smrti v dôsledku paralýzy dýchacích svalov.

V bakalárskej práci sú zozbierané najnovšie informácie z dostupných literárnych zdrojov o najnovších poznatkoch o botulizme. Dôraz je kladený na výskyt rôznych typov pri botulizme, epizootológie a epidemiológie, patogenézy, klinických príznakov a stanovenia diagnózy. Na konci práce sú uvedené najvýznamnejšie preventívne a kontrolné opatrenia vykonávané v populácii zvierat a ľudí.

## Abstract

Botulism, a toxic infection, is usually a foodborne disease that is common to both humans and many animal species. The disease is caused by the consumption of food that has been contaminated with performed botulinum toxin or *Clostridium botulinum* spores. Outbreaks are always very serious and considered medical emergencies. Prompt recognition of the clinical symptoms and rapid investigation of the potential source of contamination is crucial to prevent additional cases and to enable early administration of antitoxin. Without rapid treatment, death is most likely to occur from paralysis of the respiratory muscles. In this bachelor thesis, the objectives are to collect information from literature sources to summarize the latest knowledge about botulism. The focus is on the occurrence of the different types of botulism, the epizootiology and epidemiology, pathogenesis, clinical signs and diagnosis. The most important preventive measures and how botulism is controlled in animal and human populations are emphasized at the end of the thesis.

## Contents

## List of tables and figures

## List of abbreviations and symbols

a) 4-AP - 4-aminopyridine
b) BoNT – Botulinum neurotoxin
c) CDC - Centers for Disease Control and Prevention
d) CFSPH - Center for Food Security and Public Health
e) CSF – Cerebrospinal fluid
f) EFSA – European Food Safety Authority
g) E.g. - *Exempli gratia* (for example)
h) ELISA - Enzyme-linked immunosorbent assays
i) GBS - Guillain-Barré syndrome
j) LEMS - Lambert-Eaton myasthenic syndrome
k) MG – Myasthenia gravis
l) MLD - Minimal lethal doses
m) NIAID - National Institute of Allergy and Infectious Diseases
n) PCR - Polymerase chain reaction
o) SMA - Spinal muscular atrophy
p) SNAP - Synaptosomal-associated protein
q) SNAP-25 - Synaptosomal associated protein 25
r) SNARE - Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
s) SVA – Statens Veterinärmedicinska Anstalt
t) VAMP - Vesicle-associated membrane protein
u) VAMP - Vesicle-associated membrane protein
v) WHO – World Health Organization

# 1 Introduction

Botulism is a rare but serious paralytic illness caused by nerve toxins that are produced by the rod-shaped, spore-forming and anaerobic bacterium *Clostridium botulinum*. The botulotoxin is of the most potent toxin known. They bind to nerve endings where they block the release of acetylcholine to motor neurons, leading to progressive flaccid paralysis in both humans and animals. Without rapid treatment, death is most likely to occur from paralysis of the respiratory muscles.

*C. botulinum* spores are commonly found in the environment, especially in soil, sediments and waters, but can germinate and grow only under specific conditions. Sporadic cases and outbreaks occurs, and often affects a whole livestock due to a common source of contamination, causing significant economical losses. In areas where botulism is prevalent, vaccines may be used in animals including horses, cattle, sheep, goats, mink and birds.

In humans, botulism is typically caused by improperly preserved food. This foodborne type of botulism, is the most common type in humans. The intoxication is often due to failure in the heating process during food processing. If the food is insufficiently heat treated, the spores will survivie and production of toxins in the food is likely to occur. Other factors influencing the survival of bacterial spores in food includes pH, water activity, atmosphere and preservatives.

Infant botulism is another type of botulism in humans. Here, *C. botulinum* spores are ingested, and then germinates in the intestinal tract where they release toxins. The infant intestinal tract lacks natural defense systems, that are normally developed in most adults. A third type of botulism in humans is wound botulism, which occurs when open wounds or injured tissue becomes contaminated with *C. botulinum* spores that proliferate and produce the toxin locally. In addition, these very potent neurotoxins are a concern in bioterrorism (Coleman and Yergler, 2002 ; CFSPH, 2010 ; Folkehelseinstituttet, 2013).

Outbreaks of botulism is always very serious, and considered medical emergencies. Prompt recognition of the clinical symptoms, and rapid investigation of the potential source of contamination is crucial. This thesis mainly explains the different types of botulism, their cause, distribution and how the disease can be prevented from a public health point of view.

# 2 The state of knowledge at home and abroad with respect to the field involving the theme of the study

Botulism, a toxic infection, is usually a foodborne disease that is common to both humans and many animal species. The disease is caused by the consumption of food that has been contaminated with performed botulinum toxin or *Clostridium botulinum* spores (Akakpo and Bonfoh, 2010).

## 2.1 Historical background

At the end of 17th century and the beginning of the 18th century, a large number of sausage poisoning was reported in southern Germany due to a locally popular type of blood sausage. The German poet and physician Justinius Kerner (1786-1892) started to investigate the cases. He accumulated all the information he could on the outbreaks, and also experimented widely. The term botulism was coined, from the Latin for sausage *botulus*, for the condition often known then as Kerner's disease (Smith, 1977). Later, in 1895, the Belgian bacteriologist Emile van Ermengem (1851-1922) was summoned to the Belgian town of Ellezelles. Here, 23 musicians from a funeral had developed botulism after consumption of raw ham (Cherington, 1998). Those who had eaten smaller amounts had milder symptoms and survived, three died. Van Ermengem isolated an anaerobic bacterium from the ham. From the toxin secreted by the organism, he produced the disease in laboratory animals by injection of the toxin. The animals showed similar clinical signs as botulism in men. The bacterium was named *Bacillus botulinus* (Smith, 1977).

In the following years, the potentially botulinogenic foods were thought to be only meat or meat products. But an outbreak in Darmstadt, Germany, caused by canned white beans led to new information about the bacteria. The bacteria isolated from the Ellezelles ham and from the Darmstadt beans were compared at the Royal Institute of Infectious Disease in Berlin. They found out that the two strains produced toxins of very similar properties, but the strains differed in cultural characteristics. Also it was found that antitoxins against one culture did not have effect on the other culture, hence there was more than one kind of *Bacillus botulinus*. Georgina Burke gave the two strains their alphabetic designations; Ermengem's strain belonged to type B and the strain from the Darmstadt outbreak belonged to type A.

*C. botulinum* type C was discovered some years later – in the United States in 1922. It was found in green fly larvae involved in paralytic diseases of chickens. The same year in Australia it was found out that the disease of cattle, then called *bulbar paralysis,* was really an intoxication due to meat contaminated with a related organism to that found in the green larvae. These two were pointed out as two subtypes, - *Clostridium botulinum* type C alpha and beta (Smith, 1977).

*C. botulinum* type D was isolated in 1928 and was associated with bovine paralysis ("Lamsiekte"), responsible for the death of thousands of cattle in South Africa (Akakpo and Bonfoh, 2010).

Between 1936 and 1937 an isolated strain from fish, which was involved in an outbreak of botulism in Ukraine, became designated as type E of *C. botulinum*.

Type F was recognized by Moller and Scheibel in 1960. It was isolated from homemade liver pie that had caused an outbreak of botulism in Denmark (Smith, 1977).

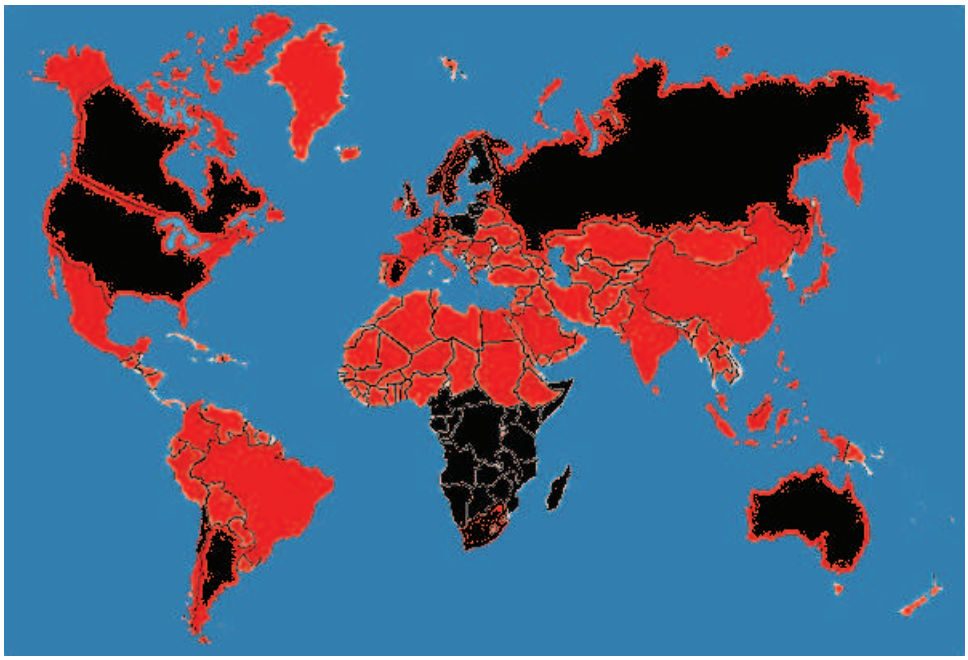
The last type of *C. botulinum* is known as type G, or also *C. argentinense*, and was described by Gimenez and Ciccarelli in 1970 in Argentina. Its origin is soil, and it has never been demonstrated in foods (EFSA, 2005).

## 2.2 Geographic distribution

Botulism is a ubiquitous disease being present on all continents. The spores are widely distributed in the soil, sea sediments, lakes and coastal waters, and in the intestinal contents of fish and other animals. Environmental factors can influence where botulism is seen. For example phosphorous-poor soils in southern Africa makes the disease common in these areas (CFSPH, 2010). Soils in Europe typically contain type B toxins, while type E is especially common in fresh water and marine sediments. In the United States, the soil in western states is commonly contaminated with type A, the eastern states with type B. Type E toxins are most common in the north, in for example Alaska, where the marine environment is contaminated and there are also long traditions with preparing fermented fish (EFSA, 2005). The disease develops rapidly to death in both humans and animals, for example did 15,000 animals died from botulism in five years in Brazil. Knowing the toxin types that are prevalent in an area is very helpful in selecting an antitoxin (Akakpo and Bonfoh, 2010).

The countries shown in black have recorded incidences of outbreaks of botulism.

*Figure no. 1 Recorded incidences of botulism*



*Source: Phil Strandwitz, 2008*

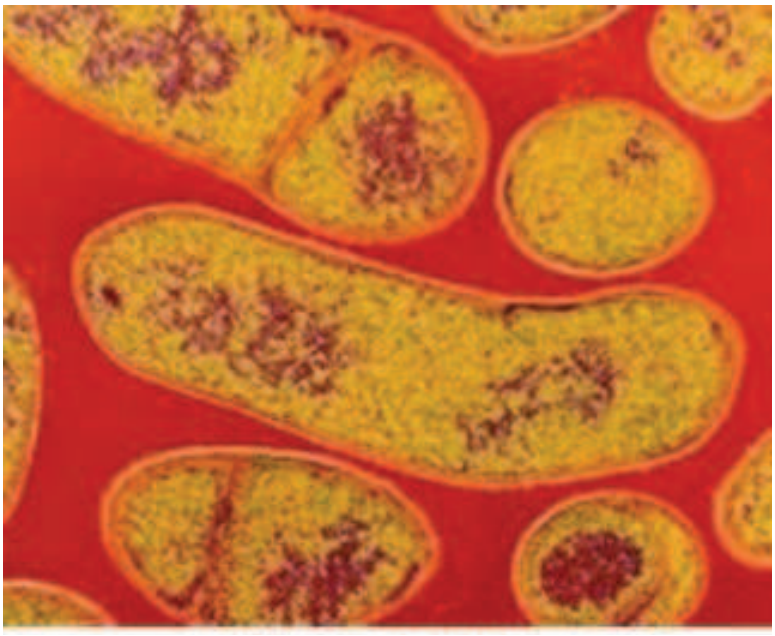
## 2.3 Etiology

Botulism is caused by botulinum neurotoxin, produced by the anaerobic, spore-forming bacterium *Clostridium botulinum*. Botulism in humans is usually caused by toxin types A, B and E. In rare cases also type F and G has been described. Types C and D are the most common causes of the disease in animals (CFSPH, 2010).

The genus *Clostridium* embraces several species, found in many places in the environment, most notably the soil. The members are all characterized by being gram-positive, spore-forming anaerobs producing extracellular toxins. Their size varies between 0,2-4 μm and 2-20 μm, and their ability to form spores is crucial to withstand the intestinal environment (McVey *et al.,* 2013).

*C. botulinum* is the species responsible for causing botulism. It is a rod-shaped bacterium with round extremities and is about 4-6 μm long and 0,8-1μm wide. The bacteria are motile by peritrichous flagella arranged singly, in pairs or in small chains. *C. botulinum* develops into an ovoid spore that is highly resistant in the environment. Being an obligate anaerobic bacterium, *C. botulinum* requires an oxygen-free environment for its growth. Optimum temperature is approximately 25$^{o}$C (range: 16$^{o}$C-40$^{o}$C), with some exceptions between strains. Optimum temperature for toxin production ranges from 25$^{o}$C to 33$^{o}$C. The bacteria prefer a slightly alkaline environment (pH 7.0-7.6) (Akakpo and Bonfoh, 2010 ; Smith, 1977).

*Figure no. 2 Clostridium botulinum*



*Source: http://www.physio-pedia.com/Botulism [20.03.2014]*

*C. botulinum* is classified as a single species, but is divided into four phenotypic groups (Group I – IV) based on the ability to digest complex proteins. The bacteria produce eight different neurotoxins (A, B, Cα, Cβ, D, E, F, G), the most potent toxins known (EFSA, 2005 ; Sykes, 2014).

*Table no. 1 Groups and types of C. botulinum*

| *Group* | *Characteristics* | *Botulinum neurotoxin (BoTN)* |
|---|---|---|
| I | Proteolytic | A, B, F |
| II | Non-proteolytic | B, E, F |
| III | | Cα, Cβ, D |
| IV | | G |

*Source: Self-made table*

All eight types of botulinum neurotoxin (BoNT) are zinc endopeptidases with the same hydrolytic acticity on the docking proteins required by neurotransmitter-containing vesicles to fuse with the presynaptic membrane. The result of the hydrolysis is blockage of the release of neurotransmitters (acetylcholine) and this is the same result for all the toxin types. What distinguishes the toxin types is that they hydrolyze different docking proteins. *C. botulinum* type A and E hydrolyze SNAP-25 (synaptosomal-associated protein 25). Toxin types B, D, F and G hydrolyze VAMP (vesicle-associated membrane protein, also known as synaptobrevin). Type C hydrolyzes SNAP-25 and syntaxin. A hydrolyzed synapse requires weeks to months to regenerate. The botulinum toxins consist of two polypeptide chains, a heavy chain and a light chain linked by a disulphide bond. The light chain includes zinc peptidase activity. The heavy chain has a translocation domain being responsible for the formation of a pore through which the light chain passes, and a binding domain for binding to nerve cells. Although the toxin types have very similar physical and chemical properties, they differ greatly in toxicity for different animal species (McVey *et al*., 2013 ; Sykes, 2014 ; Maselli and Bakshi, 2000).

In recent years, type A botulinum toxin has been used in the treatment of spasmodic torticollis. This muscle disorder is characterized by the neck muscles contracting involuntarily, causing abnormal posture of the head and neck. Controlled amounts of the toxin are injected directly into two or more neck muscles. Due to the blockage of transmission of acetylcholine, the muscles relax. After repeated treatments, the muscle tension will eventually return to its normal level. Botulinum toxin is concluded to be the most effective available therapy for spasmodic torticollis (Granum, 2007 ; Anderson *et al*., 1992 ; Poewe *et al*., 1992).

Botox is also a very known cosmetic product, where small amounts of botulinum toxin type A are injected into the tissue to smooth out the skin (Granum, 2007). It is used in treating frown lines by blocking the transmission of neural impulses to muscles, thereby weakening them and eliminating wrinkles. The effect lasts for about four months. Botox has got many fans, and only in America more than 1.6 million people injected the toxin in 2001. The most common side effects include temporary ptosis, headache, flu, nausea and facial pain and swelling (Coleman and Yergler, 2002).

### 2.3.1 Affected species and susceptibility

Performed toxins in various sources including decaying vegetable matter (grass, hay, haylage, spoiled silage, grain), fish, meat, carcasses and contaminated water, can cause botulism in animals. Susceptible animals are herbivores such as horses, sheep, cattle and goats and they usually become ill when they are fed with forage (hay or insufficiently acidified silage) contaminated with the toxin. Also birds, fish and fur animals (fox, otter, and mink) are susceptible. In experimental conditions, guinea-pig and mouse are highly susceptible and are used as laboratory animals for laboratory diagnosis (Akakpo and Bonfoh, 2010 ; CFSPH, 2010)

Horses and mules were the animals first described as suffering from botulism by Buckley and Shippen in 1917 (Smith, 1977), and botulism is more frequent in horses than any other herbivore. Horses grazing pasture after a dry period can develop a condition known as grass sickness. The exact cause of this condition is not known, but the clinical signs includes cervical weakness, muscle tremors and lateral recumbency, and there is evidence that botulinum toxin is involved due to detection of large amounts of *C. botulinum* toxin type C in the gut of animals suffering from grass sickness (Ostrowski *et al*., 2012 ; McDonald *et al*., 2011).

Nutritional factors may lead to botulism in ruminants. Cattle in those portions of the world where the soil is phosphorous-deficient may develop pica where they will eat various objects that ordinarily they would ignore, such as carcasses of small animals (Smith, 1977). Only a gram of meat, if it is contaminated, will contain enough botulinum toxins to kill a cow. Similar cases are seen in Australia, where protein-deficient sheep sometimes eat the carcasses of rabbits and other small animals. Wild birds may ingest botulinum toxins in maggots that have fed on contaminated carcasses, or they can become ill by ingestion of contaminated fish (CFSPH, 2010). In addition, some cases of botulism results from animals drinking water that has become toxic from the decomposition of the carcasses of buried animals (Smith, 1977).

In pigs and domestic carnivores, botulism is rare. The disease in dogs may occur in hunting dogs, where waterfowl is the carrier. The only report of botulism in cats was an outbreak that occurred after some cats were fed pelican carrion. Botulism in dogs and cats is almost always caused by ingestion of botulinum toxin type C (Sykes, 2014). According

Jane E. Sykes: "The relative resistance of dogs and cats to botulism has been hypothesized to reflect an adaptive response to a carnivorous lifestyle" (2014:520). Botulism in pigs is also very rare, though the eating habits of unconfined pigs should make them good candidates for the disease. It appears that the gastrointestinal tract of pigs may have a low permeability for botulinum toxin. But there are a few reported outbreaks of botulism in pigs due to consumption of dead fish and decomposing brewers waste (Zimmerman *et al*., 2012). These outbreaks have been caused by ingestion of botulinum toxin type C. Cold-blooded animals are not receptive for botulism (Akakpo and Bonfoh, 2010).

### 2.3.2 Pathogenicity and resistance

*C. botulinum* is an alimentary bacterium where botulism can be the result from the ingestion of preformed toxin. As mentioned, the toxins produced are of the most potent toxins known. One milligram contains 4,800,000 minimal lethal doses (MLD) for guinea pigs. In mice, the MLD of type A toxin is 1.2 nanogram per gram body weight by intraperitoneal route (Akakpo and Bonfoh, 2010 ; Sykes, 2014). Table 2 below shows MLD of different types in mice.

*Table no. 2 Minimal lethal dose of different types of toxin in mice*

| *Type of toxin* | *MLD/g live weight (ng)* | *Route of inoculation* |
|---|---|---|
| *A* | 1.2 | Intraperitoneal |
| *B* | 1.2-2 | Intraperitoneal |
| *C* | 1.1 | Intravenous |
| *D* | 0,4 | Intraperitoneal |
| *E* | 1.1 | Intraperitoneal |
| *F* | 2.5 | Intraperitoneal |

*Source: adapted from Akakpo and Bonfoh, 2010.*

The vegetative form of *C. Botulinum* has low resistant to the environment. However, the spores produced are considered to be the most resistant bacterial spores. They can withstand alcohol, quaternary ammonium, phenol compounds and ultraviolet rays. They are not resistant to gamma radiation, formaldehyde, ethylene, propylene or heat. Group 1 spores are inactivated after heat treatment at 121$^{o}$C for 3 minutes, Group 2 spores after 10 minutes at 90$^{o}$C. The pH, water activity and fat content of the food must also be

taken into consideration concerning the heat resistance of the spores. Spores are found more heat resistant at low water activity and neutral pH values, and more sensitive at low pH values (EFSA, 2005). The resistance explains the importance of heat processing in the preparation of preserved foods.

In botulism outbreaks, toxin is released in tissues of the organism. Animals can be protected against the disease if antitoxins are produced by their immune system after initial exposure to the sepcific toxin type. It is necessary to select antitoxins of the serotypes that are present in the particular region for successful treatment or vaccination (Akakpo and Bonfoh, 2010).

## 2.4 Epizootiology and epidemiology

*C. botulinum* produce spores that are highly resistant to disinfectants, heat and environmental factors that kill the vegetative cells (CFSPH, 2010). The botulinum spores can be found in soil, waters, and the digestive tract of animals. The spores that are found in the digestive tract of animals increase the risk of human contamination after consumption of e.g. sausages prepared from intestine of infected animals. Again, the heat processing in the preparation of food is crucial to avoid the risk of contamination. Carcasses of infected animals may cause a huge risk of contamination of the local environment as the putrefaction facilitates the germination of the spore and its transformation into the vegetative form which produces the toxin. If the carcass fall into a water hole, or the putrefaction process occur in the field where forage is made, both food and water source will be contaminated (Akakpo and Bonfoh, 2010). Invertebrates such as snails, earthworms and maggots that are feeding on contaminated carcasses are important in transmitting the toxins into species such as birds. Invertebrates appear to be unaffected by the toxin (CFSPH, 2010). The presence of spores of *C. botulinum* has not been reported in herbs and spices (EFSA, 2005).

Botulism outbreaks differ in different countries reflecting the distribution of *C. botulinum* in the soil and the different patterns of food production, processing and preservation (EFSA, 2005). Botulism is usually associated with traditionally made meat and fish. Especially home-smoked and fermented fish, or home made sausages prepared from intestine of infected animals (Akakpo and Bonfoh, 2010). In addition to meat and fish, botulinum toxins can also be found in for example bottled chopped garlic, baked

potato, home preserved asparagus and other home-canned vegetables (Solomon and Kautter, 1988 ; Angulo *et al*., 1998 ; Paterson *et al*., 1992). In Norway, the first incidence of botulism in humans was described in 1934, due to traditionally made ham. Since then, there have been only 5 deaths among the 160 reported cases. The major source has been the traditionally prepared "rakfisk" (Granum, 2007). Rakfisk is semi-fermented fish where salt and sugar are added to a gutted fish, which is put into a pressurized container and stored for several weeks at 5-8°C before being eaten without cooking. If the temperature during fermentation gets higher than 8°C and/or too little salt is added, *C. botulinum* may germinate and produce toxin (Eriksen *et al*., 2004).

*Table no. 3 Food involved in outbreaks of botulism*

| | Number of outbreaks with food identified | Meats (%) | Fish (%) | Fruit and vegetables (%) | Other (%)[a] | Home prepared (%) |
|---|---|---|---|---|---|---|
| *USA* | 222 | 16 | 17 | 59 | 9 | 92 |
| *China* | 958 | 10 | 0 | 86 | 4 | |
| *Argentina* | 14 | 29 | 21 | 36 | 14 | 79 |
| *Poland* | 1500 | 83 | 12 | 5 | 0 | 75 |
| *Czechoslovakia* | 14 | 72 | 7 | 14 | 7 | 100 |
| *Hungary* | 28 | 89 | 0 | 4 | 7[d] | 100 |
| *Norway* | 19 | 16 | 84 | 0 | 0 | 100 |

[a]Including mixed vehicles. [b]Mixtures of meats and vegetables

*Source: adapted from Hauschild and Dodds, 1993*

Contamination by the toxin is mostly indirect intoxication after ingestion and absorption of toxin-containing material; botulism develops when animals ingest preformed toxins in food. Toxin-containing materials can be water and decaying vegetable matter

(grass, grain, hay, spoiled silage or cereal contaminated by animal carcasses) (Akakpo and Bonfoh, 2010). If chicken litter, which may contain dead chickens contaminated with the toxins, is spread as fertilizer onto pastures where ruminants graze, this can be a source of outbreak. Increased use of big bale silage and round hay baling in which grass is ensiled without acidification in airtight bags has increased botulism outbreaks in cattle and horses the recent years. It is likely that it is due to the combination of anaerobic conditions and cutting the grass close to the ground so that birds and rodents may be collected (McVey *et al*., 2013). In carnivores, especially mink, botulism is caused by type C strains that have produced toxins in chopped raw poultry meat or fish that make up a large portion of their feed (Kahn, 2010).

What has being described above is the intoxication route of botulism. Another route in which botulism can develop is from ingestion of spores that will germinate in anaerobic tissue and produce toxins as they grow (toxicoinfectious botulism) (Whitlock and McAdams, 2006). Toxicoinfectious botulism occurs predominantly in foals and is known as shaker foal syndrome (SVA, 2014). The normal intestinal flora of adult horses, human and other animals will inhibit the growth of ingested botulinum spores (Whitlock and McAdams, 2006). Shaker foal syndrome is similar to the infantile botulism in human. In rare cases, wound botulism is seen in some animal species such as horses where *C. botulinum* contaminates the umbilical region in newborn foals (CFSPH, 2010).

In humans, the three main forms of botulism are foodborne, infant and wound botulism. Foodborne botulism occurs after ingestion of preformed toxin produced in food by *C. botulinum* (Shapiro *et al*., 1998). Improperly preserved food is usually the source because the bacterial spores are resistant to heat and may survive home-canning processes if temperatures are below $120^{o}$. Environmental factors that favor spore germination and toxin production are low acidity, low oxygen and relatively high water content (Townes *et al*., 1996). Boiling of the food should destroy the toxin. The ingested neurotoxins are resistant to the proteolytic acticity within the stomach because accessory proteins protect them from proteolysis (Sharma and Singh, 1998). The alkaline environment of the intestine ensures the dissociation from these accessory proteins, and the toxins are absorbed and transported via the circulation to nerve terminals of skeletal muscles and the peripheral autonomic system. Although the clinical signs for each toxin type can be similar, it has been observed that cases caused by toxin type A may be more severe and last longer. The

fatality rate of foodborne botulism is approximately 20%, and the first symptoms are dry mouth and blurred vision (Maselli and Bakshi, 2000 ; Cherington, 1998). The vulnerability to these neurotoxins varies among individuals due to different genetic backgrounds of the susceptibility (Thajeb *et al.*, 2007).

The infant variant of botulism was first described in 1976 (Maselli and Bakshi, 2000). The United States sees approximately 90% of infant botulism cases worldwide, reporting about 110 cases annually (Brown and Desai, 2013). Most cases occur before the age of 6 months. In foodborne botulism, preformed toxin is ingested trough which food is contaminated. In infant botulism, *C. botulinum* spores are ingested. These then germinates the intestinal tract of the infant where they release toxins. In most adults and older children, natural defenses are developed over time to prevent germination of the bacterium. The infant intestinal tract lacks both the protective bacterial flora and the clostridium-inhibiting bile acids that are normally a part of the natural defense system of adults (Cherington, 1998 ; Walker, 2004). Constipation is usually the first clinical signs. Up to 15% of the cases of infant botulism, the ingestion of honey is suspected. Microbiologic survey of honey products has reported the presence of *C. botulinum* spores in up to 25% of products. For this reason, honey should not be given to children during the first year of life (Schmidt and Schmidt, 1992 ; Shapiro *et al*., 1998 ; Spika, *et al*., 1989).

Wound botulism occurs when open wounds or injured tissue becomes contaminated with *C. botulinum* spores that proliferate and produce the toxin locally (Davis *et al*, 195). It can develop from childbirth or improperly treated open fractures if the environment is contaminated (Walker, 2004). Most cases have been reported in the United States, and so far only types A and B have been encountered. In the recent years, most cases of wound botulism have been reported in drug addicts in association with subcutaneous injection (Maselli and Bakshi, 2000). In October and November 2013, four cases of wound botulism were reported in people who inject drugs in Norway. There were two men and two women between the ages of 35 and 55. They were all mentally alert and had classic signs and symptoms of botulism, including ptosis, ocular muscle paralysis and dry mouth. They were placed on a mechanical ventilator due to respiratory failure and treated with botulinum antitoxin (MacDonald *et al*., 2013).

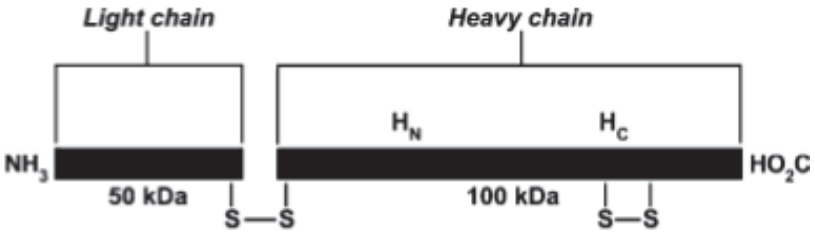
There is a fourth type of botulism, called inhalation botulism. It is a very rare type of the disease and does not occur naturally – it is often associated with bioterrorism (WHO, 2013). Botulinum toxin is the most poisonous natural substance known, and it is on the

Centers for Disease Control and Prevention (CDC) list of biologic agents of highest concern as a biological weapon (CDC, 2001). The toxin has previously been produced as a biological weapon by several nations, including Iraq, Japan, United States and the Soviet Union (Coleman and Yergler, 2002 ; Folkehelseinstituttet, 2013). In 1972, research and manufacture of biological weapons became strictly prohibited (Coleman and Yergler, 2002). However, Iraqi officials have admitted producing 19,000 liters of botulinum toxin, using half of that in military weapons. This amount is three times the amount required to kill every human being on earth (Arnon *et al.*, 2001).

## 2.5 Pathogenesis

*C. botulinum* toxins are either directly ingested as preformed toxins, or later produced by the bacteria as they grow within the body in anaerobic tissue. The portal of entry is most commonly through the alimentary tract, in rare cases through the pulmonary tract (inhalation botulism) or mucus membranes of wounds (Smith, 2009). In intoxication, the toxin will withstand the acid environment of the stomach. As it enters the small intestine, digestive enzymes (e.g. trypsin) or bacterial protease will react on the toxin transforming it into active form. The active toxin is absorbed into the blood stream and travels to peripheral cholinergic synapses, which include the neuromuscular junction and autonomic synapses (Akakpo and Bonfoh, 2010 ; Sykes, 2014). Each of the botulinum toxin types consist of two polypeptide chains, a 100-kd heavy chain joined to a 50-kd light chain by a disulphide bond (Hatheway, 1990 ; Dressler, 2012). The light chain is globular protein with zinc endopeptidase activity in which it cleaves neuronal SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins involved in synaptic transmission. The heavy chain has two domains: the N-terminal translocation domain and the C-terminal receptor-binding domain. The translocation domain consists of long $\alpha$-helices that facilitates the passage of the light chain into the cytosol of the nerve cell. The receptor-binding domain is important for the association of the toxin with the target cell. Variation in the receptor binding affinity may explain the differences in species susceptibility to *C. botulinum* toxins (Karalewitz and Barbieri, 2012 ; Sykes, 2014).

*Figure no. 3 Structure of botulinum neurotoxin*




*Source: Smith, 2009.*

After binding to nerve cell receptors, the toxin light chain is internalized through receptor-mediated endocytosis. Once inside the nerve terminal, the light chain hydrolyzes the SNARE proteins. Different botulinum toxin types hydrolyze different SNARE proteins: toxin type A and E hydrolyze SNAP-25 (synaptosomal associated protein), type B, D F, and G hydrolyze VAMP (vesicle-associated membrane protein, also known as synaptobrevin) and type C hydrolyzes both SNAP-25 and syntaxin (Maselli and Bakshi, 2000). The SNARE proteins constitute the core of the vesicular fusion machinery enabling fusion of neurotransmitter vesicles with the neuronal plasma membrane and permits release of acetylcholine into the synaptic cleft (Smith, 2009). This cause depolarization of the muscle membrane and muscle contraction in normal physiological conditions (Akakpo and Bonfoh, 2010). Cleavage of these proteins under pathological conditions prevents membrane fusion and release of acetylcholine from the nerve cell. The nerve impulse stops, and one of the first clinical signs is acute flaccid paralysis (Schiavo *et al*., 2000 ; Simpson, 2004). When this affects respiratory muscles, death due to respiratory failure occurs (McVey *et al*., 2013).

## 2.6 Clinical signs

### 2.6.1 Clinical signs of animals

The incubation period between consumption of toxic material and onset of signs is largely determined by the amount of toxin consumed, and also according to the susceptibility of the subject (Akakpo and Bonfoh, 2010). It can be 2 hours to 2 weeks, in most cases the first clinical signs appear within 12 to 48 hours (CFSPH, 2010). When the duration of incubation is shorter than 24 hours, the disease is fatal. Longer incubation time gives better prognosis (Akakpo and Bonfoh, 2010). The clinical signs of botulism are

neuroparalytic. According to Akakpo and Bonfoh (2010), the signs are variable depending on the animal species but can be divided into four major groups: muscle paralysis, secretion deficiency, visual disturbance and negative signs. The negative signs (absence of some clinical signs that are specific of other diseases) are constant in all affected species; no fever is seen, and there is usually no modification of cardiac and respiratory rhythm in the first stages of the disease. Botulism has been reported in a variety of vertebrates including horses, cattle, sheep, birds and fish, as well as in farmed mink and foxes. Is has also been documented in laboratory animals and some wild species. Dogs, cats and swine are relatively resistant to the disease, but some outbreaks have been reported (CFSPH, 2010).

***Cattle***

Cattle usually contract botulism in two ways (Smith, 1977). In areas with phosphorous deficiency, cattle may develop pica, chewing the bones of carcasses of animals that died of botulism and thus ingesting the toxin (Gyles *et al.,* 2010). Another possible way for cattle to be infected is by eating contaminated feed. Some cases of disease have been as a result of drinking contaminated water. At the onset of disease, the most apparent sign is muscular incoordination progressing to paralysis. Weakness is seen in the hindlegs first, gradually spread to the motor muscles of the forelegs, head and neck. The animal shows difficulty to move, its walk is shaky and uncertain before it is unable to stand because the muscles are soft and flaccid. Chewing and swallowing difficulties is also typical, as well as loss of tonicity of lip muscles and tongue resulting in hanging lips and lingual protrusion. Loss of auricular muscular tonicity results in hanging ears. Restlessness and oliguria can also occur. Secretion deficiency is typical, poor production of saliva and other oral secretions results in dryness of oral cavity. In some cases visual disturbance is seen with mydriasis and loss of light reflex followed by blindness. When lateral recumbent animals are seen, they are usually very close to death and their head is often turned toward the flank as in cows suffer from hypocalcemia (CFSPH, 2010 ; Akakpo and Bonfoh, 2010). According Akakpo and Bonfoh (2010), the mortality rate is 80-85% in ruminants.

***Horses***

Characteristic early signs of botulism in adult horses include decreased tongue strength and dysphagia. These sign typically occur before onset of obvious muscle weakness. Easy tests can be done by the owner to check symptoms. A tongue test can be

done to assess the tongue strength. It is done by pulling the tongue out through the interdental space while the jaws remain closed. Let the tongue hang down. Under normal conditions, the horse will quickly retract the tongue. Under pathological conditions, the horse will retract the tongue very slowly or if at all. This tongue stress test represents one of the most sensitive and early signs of botulism in horses. A grain test can be done to assess the horse's swallowing abilities. The test is performed by offering the horse grain in a bucket and the horse's ability to consume the feed is timed. Diseased animals will consume the feed more slowly than usual and grain mixed with saliva often falls out of the mouth while eating. As the disease progress, other signs appear including muscle weakness (Whitlock and McAdams, 2006). Affected horses typically drag their hooves or stumble. Their facial expression is lacking and they appear sleepy while remaining alert (Gyles *et al*., 2010). If untreated, botulism will always lead to recumbency followed by death due to respiratory failure (Whitlock and McAdams, 2006). Foals are lying down more than normal when suffering from shaker foal syndrome. When forced to rise up, they only stand for a few moments and develop muscle tremors (hence the name) and drops down in lateral recumbency (Wichtel and Whitlock, 1991). In addition, affected foals often drools milk when suckling the mare and shows slow retraction of their tongue (Whitlock and Buckley, 1997). The shaker foal syndrome is usually seen in animals less than 4 weeks, and as in adult horses death occurs due to respiratory failure (Whitlock and McAdams, 2006).

***Birds***

Insect larvae ingest botulinum toxin when feeding on carcasses of dead animal that died from botulism. When wild birds consume these larvae, they will also be infected by the disease (Gyles *et al*., 2010). Botulism in birds has often been named “limberneck” as the first symptom of the disease is the drooping head posture, followed by total paralysis. The drooping head often causes waterfowl to drown (McVey *et al*., 2013). Both botulism in wild birds and poultry is caused by *C. botulinum* type C. Uptake of preformed toxin through the mentioned larvae on carcasses is seen as an important factor behind major epidemics among wild birds. Consequently, the role of carcasses has for long time been suspected also in poultry botulism where the idea has been that *C. botulinum* can be recovered from the intestinal tract of healthy flocks. Due to little information on the prevalence of *C. botulinum* spores within healthy poultry flocks, the Norwegian Veterinary Institute in collaboration with the Norwegian School of Veterinary Science and the

National Veterinary Institute in Uppsala recently had a project to test the hypothesis that *C. botulinum* is frequently present in healthy broilers. Their findings indicated that toxigenic spores are not normally present in broiler flocks without recent or concurrent outbreaks of botulism; hence the carcasses will only be a risk factor for outbreak if the flock is infected. The sources and vehicles for introducing the organism into poultry flocks remain unidentified (Hardy and Kaldhusdal, 2013).

***Fur animals***

In fur animal production, botulism has been a major hazard for decades (Myllykoski *et al.,* 2010). Minks and ferrets are highly susceptible to botulinum toxins, type C toxin in particular, and farmed mink are therefore vaccinated in young age. Unlike minks, foxes were thought to be resistant to botulinum toxins, until the largest reported botulism outbreak in fur animals occurred in Finland where more than 52, 000 foxes died in 2002. Botulism outbreaks in fur animals are typically caused by contaminated feed components improperly chilled or non-acidified. The feed components are mainly poultry and fish by-products. Clinical signs in diseased animals are similar to most other species and include paralysis of the hind legs, total paralysis, and recumbent position (Yndestad *et al*., 1977 ; Lindstrøm *et al*., 2004). Usually, most mink are found dead within 24 hours (Kahn, 2010).

***Domestic carnivores and swine***

Dogs and cats seem less sensitive to botulinum toxin, but a few cases of intoxication have been reported. Severely affected dogs are quadriplegic with their voluntary motor activity being absent. They are unable to rise from lateral recumbency, while their ability to wag their tail is retained. Other clinical signs include vomiting and suppressed bark. As in dogs, the single outbreak described in cats also included quadriplegia, combined with hypothermia, lethargy, dehydration and decreased segmental reflexes. According to Zimmerman *et al*. (2012), it appears that the gastrointestinal tract of pigs may have a low permeability for botulinum toxin and hence botulism in pigs is rarely seen. But there are a few reported outbreaks of botulism in pigs where the clinical signs are similar to those in other species with muscle weakness and incoordination and dilatation of the pupils, among others (Zimmerman *et al*., 2012).

### 2.6.2 Clinical signs of humans

Within 12-36 hours after ingestion of contaminated food, signs and symptoms of botulism develops. As in animals, the faster onset of symptoms the more severe the disease. Since botulinum toxin attacks nerve cells, signs and symptoms are neurologic (Walker, 2004). It often begins with bilateral cranial nerve impairment which involves the muscles of the eyes, mouth, face, head and pharynx (Smith, 2009). This paralysis cause visual disturbances, including blurred vision, diplopia, ophthalmoplegia, dilatation of the pupils and ptosis. Dry mouth, dysphagia and dysarthria are also typical. These initial symptoms way be nonspecific and difficult to associate botulism. If the disease is caused by intoxication, the ingested toxins also cause nausea, vomiting and diarrhea together with the neurologic indications. The paralysis descends symmetrically to involve the respiratory muscles and the lower extremities (Smith, 2009). Diarrhea is replaced by constipation as the paralysis descends to the intestinal muscles. In severe cases, extensive respiratory muscle paralysis may lead to ventilator failure and death unless the patient receives mechanical ventilation. Ventilatory support is commonly needed for 2-8 weeks, up to 7 months in severe cases (Shapiro *et al.,* 1998). As in animals, the disease does not usually cause a fever. The incidence of botulism is low, but the mortality rate at the beginning of this century was more than 50%. The improvement in critical care has resulted a drop in the mortality rate, from 50% to under 10% (Cherington, 1998 ; Granum, 2007).

In infant botulism, constipation may be the first clinical sign, followed by poor feeding, lethargy, weak cry, poor sucking ability and general weakness of muscles characterized by a floppy head (Cherington, 1998 ; Shapiro *et al*., 1998). With adequate supportive care, most infants recover in weeks or months. The mortality rate has been reported to be 5% (Kothare and Kassner, 1995). The clinical manifestations of wound botulism are similar to those described in intoxication, except that gastrointestinal symptoms are absent. The incubations time is also longer; it may take up to two weeks before any symptoms appear. The mortality rate is approximately 15% (Shapiro *et al.,* 1998). Symptoms in inhalation botulism are similar to those seen in foodborne intoxication (WHO, 2013).

## 2.7 Macroscopic and microscopic lesions

In classical intoxication, paralysis of the legs and tongue is pathognomonic. In other cases, a suspect animal should be necropsied and examined for macroscopic and microscopic lesions (Gyles *et al*., 2010). Usually no specific lesions are constant or sufficiently significant to be caused by botulism, but the most consistent necropsy findings are pulmonary edema and excessive pericardial fluid containing free-floating strands of fibrin. Sometimes, haemorrhagic lesions of the meninges and nervous matter can be seen. The urinary bladder is commonly distended, and in horses the mucosa might appear yellowish due to destroyed liver cells (Akakpo and Bonfoh, 2010 ; Kahn, 2010). In a thoroughbred foal diagnosed with botulism, deep ulcerations was found in the non-glandular gastric mucosa at necropsy. No other macroscopic or microscopic lesions were detected. *Clostridium botulinum* type B was isolated from the gastric lesions of gastric mucosa, whereas the cecal and colonic content were tested negative. Analyses of all potential environmental sources of toxins near the feeding areas were also negative. Hence it was concluded that the gastric lesions was not due to botulism, but the opposite way - wound botulism was caused by infection of gastric mucosa lesions (Liguori *et al*, 2008)

In birds, *C. botulinum* toxins do not cause lesions, but for example the larvae which caused the disease may by found in the crop (Kahn, 2010).

Capillary dilatation due to in intoxication might be found histologically as well as haemorrhages in the liver, kidneys and brain. But as already mentioned, none of the above lesions are constant or pathognomonic (Akakpo and Bonfoh, 2010).

## 2.8 Epizootiological and epidemiological diagnosis

### 2.8.1 Clinical diagnosis

As botulism is a life-threatening condition, a rapid diagnosis is essential for successful therapy. Diagnosis is usually based on clinical history and clinical manifestations but can often be difficult because of the rare and accidental nature of infection and the lack of specific clinical signs (WHO, 2013 ; Akakpo and Bonfoh, 2010). Diagnosis is commonly made by excluding other diseases, and differential diagnosis must be preformed (CFSPH, 2010). Botulism should be suspected if sudden morbidity and

mortality are reported in enzootic areas (disease is constantly present) and the deaths are associated with paralytic signs, such as motor muscle paralysis, tongue paralysis and also secretory disorders (Studdert *et al*., 2012 ; Akakpo and Bonfoh, 2010). If botulism is suspected, early administration of antitoxin is crucial in reducing the mortality rate. Severe botulism cases require supportive treatment with mechanical ventilation which may be required for months (WHO, 2013).

### 2.8.2 Laboratory diagnosis

Apart from the clinical manifestation and history, the diagnosis is based on positive laboratory findings. If toxin-producing clostridia in the patient and/or the vehicle is detected, the diagnosis is confirmed. Laboratory confirmation including demonstration of botulinum neurotoxin in serum and feces is obligatory. The suspected food, if still available, should also be tested. The detection of *C. botulinum* in samples such as gastric and intestinal contents, wound swabs or tissues, supports the diagnosis, but should not exclusively be considered pathognomonic (WHO, 2013 ; Lindstrøm and Korkeala, 2006). Detecting circulating botulinal toxins in serum is the most conclusive evidence of botulism. To get this evidence, blood sample must be taken as fast as possible when botulism is suspected. In later stages of the disease, toxins will be attached to the nerve cells and hence not be found in the blood (Fernandez and Ciccarelli, 1999 ; Granum, 2007).

The extreme potency of botulinum neurotoxin makes it particularly necessary to ensure the safety of the laboratory workers. Despite the potency, the noncontagious *C. botulinum* is graded as a class II pathogen. Appropriate biosafety level 2 facilities and trained personnel are therefore a minimum requirement for work with this toxin. As mentioned, *C. botulinum* toxin forms a potential threat for bioterrorism, hence restricted entrance to the laboratory facilities is a necessity (Lindstrøm and Korkeala, 2006). Botulinum neurotoxin is typically detected with mouse bioassay, but enzyme-linked immunosorbent assays (ELISAs) and polymerase chain reaction (PCR) may also be used.

***Mouse bioassay***

The mouse lethality test is the most sensitive method available and the standard procedure for detection of botulinum neurotoxin. The laboratory mice are injected intraperitoneally with sample diluted in phosphate buffer. Toxins from strains of Group II *C. botulinum* require trypsin activation before analysis, as these strains lack proteolytic

activity. If the sample contains toxin, the mice develop typical signs of botulism, including muscular weakness, limb paralysis and respiratory difficulties. These symptoms usually develop within a day but may take several days to appear. To confirm that animals die due to botulism, any toxin must be neutralized with specific antitoxins. And briefly, mice injected with the neutralizing antitoxin survive, while the others develop botulism.

The injected sample used in the assay can be fecal, serum, gastric, wound, and food samples as well as supernatants from bacterial cultures. However, the assay is time-consuming and expensive and naturally presents an ethical dilemma due to the use of live animals (McVey *et al*., 2013 ; Lindstrøm and Korkeala, 2006).

***Immunological methods***

Compared with the mouse test, the immunoassays are technically simple and fast to perform. Briefly, the neurotoxin in a sample binds to a solid matrix that is usually coated with polyclonal or monoclonal antibodies against one or more toxins (sandwich ELISA). Then a secondary enzyme-linked antibody is added and will bind the toxin. Finally, a substance containing the enzyme's substrate is added causing a reaction that produces a color change in the substrate. The sensitivity is some 10- to 100-fold lower than that of the mouse bioassay. Recently, amplified immunoassay systems have been developed and equal the sensitivities of that of the mouse bioassay. But most of these are complicated, expensive and not yet available. Doellgast *et al*. (1993) developed a sensitive modified ELISA named ELCA (enzyme-linked coagulation assay) for detection of A, B and E neurotoxins. ELCA shows a much greater sensitivity than ELISA.

Disadvantages with immunological tests are that high-quality antibodies are not generally available, and inactivated toxins and genetic variation within the different serotypes of the neurotoxin may cause false-positive results. (Fernandez and Ciccarelli ; Lindstrøm and Korkeala).

***Culture methods***

The samples taken from food and feces is usually heat-treated at 80°C for 10 min. This temperature is recommended for Group I spores, but may injure Group II spores. Heating at 60°C for 10 to 20 min a safer choice for Group II spores. The heat treatment destroys competing microorganisms while allowing clostridial spores to survive. The spores are then cultivated in solid or liquid media. As solid media, blood or egg yolk agar is most

common. The egg yolk agar enables the lipase reaction typical of *C. botulinum*. Other solid media for clostridial growth are Brucella agar with 5% sheep blood and phenyl ethyl alcohol blood agar. Liquid media includes the non-selective chopped-meat-glucose-starch medium, cooked-meat medium, reinforced clostridial medium and anaerobe broths containing various combinations of tryptone, peptone, glucose, yeast extract, and trypsin. A correct incubation temperature depends of the groups, as Group I strains grow optimally at 35 to 40°C, whereas Group II strains favor lower temperatures of 18 to 25°C. However, both groups are able to grow at temperature range 30 to 37°C, hence the ability to grow at a certain temperature should not be used as a feature determining the physiological group of *C. botulinum* (Lindstrøm and Korkeala, 2006 ; CFSPH, 2010). On solid media, *C. botulinum* colonies are seen as greyish-white with an irregular edge.

If the collection of specimen has been deferred for more than two days after ingestion of the toxin, the chances of obtaining positive results is less than 30% (Cherington, 1998).

***Molecular detection***

DNA-based detection methods are sensitive, specific and rapid compared to the mouse bioassay and culture techniques. The methods include PCR and Southern hybridization, in which PCR is most commonly used. The molecular detection techniques are based on the detection of the botulinum neurotoxin gene in the sample. The activity of the gene or the toxin is not detected, and hence PCR alone is not reliable. Advantages with the molecular techniques are the mentioned rapidity, and they do not require the use of laboratory animals (Lindstrøm and Korkeala, 2006).

### 2.8.3 Differential diagnosis

***Differential diagnosis of animals***

Botulism should be the diagnosis of first choice in cases of acute flaccid paralysis in animals if their body temperature is normal – as fever usually is absent in botulism. However, differential diagnosis must be performed. Several enchepahlo-medullar syndromes tends to show similar signs, and also general vegatal intoxication may cause similar symptoms as in botulism, with nausea, vomiting, and diarrhea.

A differential diagnosis in several animals is meningoencephalomyelitis - inflammation of the meninges, brain and spinal cord. In horses, initial clinical signs can appear as lameness and vertebral instability. In cattle and dogs, typical signs similar to those in botulism like blindness, progressive weaness and ataxia are also seen. Analysis of cerebrospinal fluid (CSF) can usually reveal significantly increased protein content, and also a large number of white blood cells in the case of granulomatous meningoencephalitis (Kahn, 2010).

Rabies is an acute, progressive viral encephalomyelitis that principally affects carnivores, but can also be differentiated from botulism in both horses and ruminants (Kahn, 2010 ; Akakpo and Bonfoh, 2010). It is especially the dumb type of rabies that can have similar signs as botulism with a progressive paralysis, but again the pattern is ascending. Immunofluoresence on brain tissue allow direct visual observation of rabies antigen-antibody reactions (Kumar and Clark, 1994 ; Kahn, 2010).

Tetanus is another differential diagnosis from botulism. The neurotoxins produced by *C. tetani* are very similar in structure and function as the botulinum neurotoxins, but they differ in their clinical effects because they target different cells in the nervous system. Botulinum neurotoxins affect the peripheral nervous system, while tetanus toxin affects the central nervous system causing painful spasms and rigidity of the voluntary muscles by blocking the release of inhibitory neurotransmitters (glycine and gamma-amino butyric acid) (Kahn, 2010 ; Sykes, 2014).

Parturient paresis or milk fever in cows share symptoms with botulism. The disease is due to a sudden loss of calcium at the onset of lactation where the cows become mildly ataxic and are likely to be unable to stand if calcium therapy is not instituted. Muscle paralysis will lead to sternal recumbency, before loss of consciousness and death (Kahn, 2010).

***Differential diagnosis of humans***

In humans, differential diagnosis of botulism also comprises several other neuromuscular disorders. These includes Guillain-Barré syndrome, myasthenia gravis, spinal muscular atrophy, Lambert-Eaton myasthenic syndrome, tick paralysis and several intoxications; e.g. with organophosphate, atropine or carbon monoxide.

Guillain-Barré syndrome (GBS) is an autoimmune disorder; the body's immune system attacks the peripheral nervous system. This leads to muscle weakness which usually follow an ascending pattern, starting with the legs and thereafter sometimes affect the arms and upper body. GBS most often affects the nerve's myelin sheaths and is therefore often called a demyelinating polyneuropathy (Winer, 2001). The pattern of ascending weakness is a way in distinguishing GBS from botulism, which has a typical descending weakness. GBS also causes elevated levels of protein in the CSF, although these protein levels might be normal in the first week of the illness. The Miller-Fisher variant of GBS, is a more difficult diagnostic challenge, with ocular and bulbar abnormalities and a descending pattern of weakness. Electrophysiologic studies are in this case helpful in locating the site of lesion at either the neuromuscular junction (botulism), or at the level of peripheral nerve (GSB) (Cherington, 1998 ; Shapiro *et al.,* 1998 ; Brown and Desai, 2013).

Myasthenia gravis (MG) is an autoimmune disorder as well. It is caused by the failure of neuromuscular transmission, as the receptors for acetylcholine at the neuromuscular junction are blocked by the body's own antibodies. Clinical signs are muscle weakness, which typically increases during use of affected muscles. Weakness of ocular and bulbar muscles is usually the initial symptoms (Conti-Fine *et al*., 2006). Patients with mild botulism may mimic the signs of MG. However, compared to botulism patients, most patients which suffer from MG will show elevated acetylcholine receptor antibodies in serological tests (Cherington, 1998).

Spinal muscular atrophy (SMA) is an autosomal recessive disorder that results in degeneration of lower motor neurons of the spinal cord. It is potentially devastating and lethal, causing muscle weakness and progressive loss of movement. SMA is frequently manifesting in infancy and childhood, and must be differentiated from infant botulism (Cifuentes-Diaz *et al*., 2002). Unlike botulism, SMA rarely has pupillary or eye involvement (Brown and Desai, 2013).

The Lambert-Eaton myasthenic syndrome (LEMS) is characterized by muscle weakness initially affecting gait and autonomic symptoms including dry mouth, constipation and erectile failure. The weakness results from a reduction in the release of acetylcholine from motor nerve terminals, caused by autoantibodies against P/Q-type

voltage-gated calcium channels. These channels allow calcium entry into the nerve cells, which is required for acetylcholine release (Newson-Davis, 2004). In serological tests, high levels of calcium channel-binding antibodies can be found (Cherington, 1998).

Tick paralysis occurs when an embedded tick, usually on the scalp or behind the ears, produces a neurotoxin resulting in an ascending, symmetrical flaccid paralysis of the host. If the tick is not removed, the toxin can be fatal and the result might be death due to respiratory failure, as in botulism. Detection of the tick, and the ascending pattern of paralysis may differentiate tick paralysis from botulism (Grattan-Smith *et al*., 1997).

A variety of poisonings should also be considered when suspecting botulism, as many includes similar symptoms like muscle weakness, vision disturbances, dilated pupils, dry mouth and nausea.

## 2.9 Treatment and prognosis

As botulism is a life-threatening condition, treatment is always urgent and should be started as soon as possible after clinical diagnosis. If treatment is not applied in the earliest stages of the disease, preferably within 24 hours, the toxin already has adhered to the 'motor plate' of the muscle (Akakpo and Bonfoh, 2010). Botulinum antitoxin is the only specific treatment of botulism. Antitoxin serum is usually produced by immunizing large animals, commonly equines, with an inactivated form of botulinum neurotoxin which trigger the animals to produce antitoxins to defend itself against the disease (NIAID, 2010) Antitoxins neutralize the circulating toxins, but those already bound to the presynaptic endplates of neurons cannot be reversed (Gyles *et al*., 2010). In bovine and horses, the amount of bivalent anti C and D serum to be administered is 1-2ml, and 1ml in small ruminants. Birds should receive 0,5ml of anti C serum. This dose should be given once every 24 hour for 5 days. The anti serum is given intravenously or intramuscularly if it is a homologous serum, or subcutaneously if it is a heterologous serum (Akakpo and Bonfoh, 2010). Supportive care, included nursing and mechanical ventilation, in valuable animals are essential. If the patient can be kept alive, the nerve cells can survive by the axons regenerating new presynaptic endplates over time – might take up to several weeks or months. For example has mechanical ventilation reduced the death rate in foals. But such supportive care is impractical and/or unavailable for adult livestock animals (CFSPH, 2010

; Kahn, 2010). If antibiotics are included in the treatment, drugs that have neuromuscular blocking properties should be avoided (CFSPH, 2010). Prognosis in recumbent animals is poor. Antitoxic treatment improves the prognosis, but the results are variable. Prevention is preferred for animals in risk (Kahn, 2010 ; Akakpo and Bonfoh, 2010).

Also in humans, the major treatment for botulism is advanced medical and nursing supportive care. As mentioned, most botulinum antitoxins are of equine origin, and hypersensitivity to these is the greatest risk of treatment. Anaphylaxis occurs within 10 minutes in 2% of patients. Test doses with small amounts, or skin tests should be performed to check the sensitivity (Coleman and Yergler, 2002 ; Walker, 2004). Treatment is by intravenous injection of 20ml of antitoxin, followed by 10ml 2-4 hours later and then every 12-24 hours as needed (Kumar and Clark, 1994). Because of the risk of allergic reactions, equine antitoxin is not used to treat infant botulism. Instead, a human-derived antitoxin known as BIG-IV/Baby-BIG is produced in human donors immunized with pentavalent botulinum neurotoxin A to E. These antitoxins may also be used to treat foodborne botulism in older patients. Additional treatment in foodborne botulism may include stomach lavage, emetics, enemas and/or cathartics to reduce the amount of toxin in the gastrointestinal tract. If wound botulism is suspected, surgical debridement of the wound should be performed and antibiotics, such as penicillin, should be given. Aerobic conditions may be induced in the wound by using hydrogen peroxide or hyperbaric oxygen therapy (CFSPH, 2010). Guanidine hydrochloride and 4-aminopyridine (4-AP) improves ocular muscle and limb muscle paralysis by enhancing the release of acetylcholine from nerve terminals. Side effects of these two drugs are bone marrow suppression and nephritis (guanidine hydrochloride), and development of seizures (4-AP) (Cherington, 1998).

## 2.10 Preventive measures

The prevention of botulism is based on two major preventive methods: sanitary and medical.

***Sanitary methods***

Inappropriate treatment of the food at any stage during processing or consumption may result in increased number of cells of *C. botulinum* and toxin production (EFSA, 2005). The most important factors for controlling growth and toxin production of *C. botulinum* in foodstuffs are temperature, pH, water activity, atmosphere and preservatives (Granum, 2007).

*Table no. 4 Properties of C. botulinum I and II*

| Properties | Group I | Group II |
|---|---|---|
| Toxin types | A, B, F | B, E, F |
| Min. temperature for growth | 10°C | 3.3°C |
| Optimum temperature for growth | 35-40°C | 18-25°C |
| Max. concentration NaCl | 10% | 5% |
| Min. water activity for growth | 0.94 | 0.97 |
| Min. pH for growth | 4.6 | 5.0 |

*Source: adapted from Granum, 2007*

*Temperature*

Growth of Group I *C. botulinum* is prevented by storage below 10°C (Table no. 4). At 10°, growth may occur but it will take several weeks to produce enough toxins for intoxication. The odor of Group I *C. botulinum* is very prominent and offensive and renders the food unacceptable. Exceptions to this are traditionally fermented fish products such as the Norwegian rakfisk, where strong odors and flavors are an important part of the product. Group II *C. botulinum* is able to grow at temperatures typically found in domestic refrigerators, by having a minimum temperature at 3.3°C (Table no. 4). Group II strains produce no such prominent odor and the foods appear normal but may contain lethal amount of botulinum neurotoxin.

Heat treatment is the main process for inactivation of bacterial spores in food. Group 1 spores are inactivated after heat treatment at 121°C for 3 minutes, Group 2 spores after 10 minutes at 90°C. The heat resistance of the spores is affected by pH, water activity and fat content. For example are spores more heat sensitive at low pH values (below 4.6) and hence acid additives can be used in controlling bacterial growth (EFSA, 2005 ; Granum, 2007).

*pH*

Growth of *C. botulinum* slows as pH falls below optimum (pH 7.0-7.6). Minimum value for growth is 4.6 for Group I and 5.0 for Group II (Table no. 4). Water activity and temperature can have an effect, but only enables the bacteria to grow at higher pH values,

never lower. And as mentioned, acid additives can therefore be very suitable in controlling bacterial growth (EFSA, 2005 ; Granum, 2007).

*Water activity*

There are no growth of *C. botulinum* Group I at water activity below 0.94 or below 0.97 for Group II (Table no. 4). The water activity is reduced either by drying or by adding for example sodium chloride (NaCl). The bacterial growth stops if the concentration of NaCl exceeds 10% for Group I, or 5% for Group II (Table no. 4) (EFSA, 2005 ; Granum, 2007).

*Atmosphere*

Since *C. botulinum* is an anaerobic bacterium, it grows easily in vacuum-packed food if the temperature and pH is suitable. Even if the above atmosphere contains oxygen, the bacteria could multiply if the surface of the food is sufficiently anaerobic, usually on fish and meat. $CO_2$, which usually inhibits growth bacterial growth, stimulates the growth of *C. botulinum* (EFSA, 2005 ; Granum, 2007).

*Preservatives*

The most important preservative used to prevent growth of *C. botulinum* is nitrite. To add nitrite to salted products is permitted in certain amounts. The effect of nitrite is related to a balance between pH, temperature and time (Granum, 2007).

*Other processes*

Heating has been the main process for inactivation of bacterial spores in food, but many novel processes are being explored, such as hydrostatic pressure, pulsed electrical fields and irradiation. High hydrostatic pressure inactivates vegetative bacteria, but relies on low pH to prevent surviving of spores. The concern that some super-dormant spores may remain from this process is why using hydrostatic pressure is not used commercially (Gould, 1995 ; Ross *et al*., 2003).

Also animal food must be prepared correctly and be kept in hygienic environments. Grains, forage reserves and water reservoirs should be monitored from pollution by dead rodents. Forages contaminated by small animal carcasses or with apparent mold growth

should not be fed. Any dietary deficiencies, such as phosphorous deficiency, should be corrected by providing mineral and vitamin supplements. During an outbreak, carcasses must be collected to prevent animals from eating contaminated tissues. Flies should be controlled so the occurrence of contaminated larvae is kept to a minimum, otherwise the disease may easily gets spread to birds feeding on the larvae. Contaminated poultry litter should not be used as fertilizer (CFSPH, 2010 ; Akakpo and Bonfoh, 2010).

***Medical methods***

In areas where botulism is prevalent, vaccines may be used in animals including horses, cattle, sheep, goats, mink and birds (CFSPH, 2010). The early developed tetanus vaccine provided a good model for the work on botulism vaccine. By 1993, the nucleotide sequences for all seven structural genes encoding botulinum neurotoxin serotype A-G were known (Smith, 2009). The vaccine availability varies with the country. In Norway mink are routinely vaccinated against botulinum type C toxin, by subcutaneous injection of 1ml *C. botulinum* type C toxoid. Vaccination of cattle with types C and D toxoid has been successful in South Africa and Australia (Kahn, 2010). In Sweden, horses are commonly vaccinated with *C. botulinum* type B toxoid (SVA, 2014). There is no cross-protection between toxin types, and if an animal survives the disease it is not protected from later exposure to that toxin.

In men, only high-risk groups such as laboratory workers who work with botulism specimens, or military personnel in risk of botulism when used as biological weapons, are vaccinated (Shapiro *et al.,* 1998).

# 3 Goal of the study

The objectives of this bachelor thesis was to collect information from literature sources to summarize the latest knowledge about the major bacterial zoonosis - botulism. With a focus on the occurence, etiology, epizootiology and epidemiology, pathogenesis, clinical signs, diagnosis, prevention and control of botulism in animal and human populations.

# 4 Methods of study

The methods of study, which have been used throughout this thesis, have mainly consisted of collecting, sorting and assembling together relevant information from different literature sources. This thesis is based purely on theoretical knowledge, which has been assembled from previously published sources. The information used here, has been obtained and collected from various textbooks, journals and articles published in scientific journals (in electronic form).

Articles from electronic journals has been used to a larger extent in comparison with other sources of information, due to the ease of access and a vast selection to choose from. These articles have been found through searches in large international scientific databases, while being accessed through the online library of the University of Nordland.

# 5 Conclusion

Botulism is a rare disease, but outbreaks are always very serious and considered medical emergencies. Prompt recognition of the clinical symptoms and rapid investigation of the potential source of contamination is crucial to prevent additional cases and to enable early administration of antitoxin. Without rapid treatment, death is most likely to occur from paralysis of the respiratory muscles.

The best way to control and prevent occurrence of botulism in animal populations is by using strict husbandry methods and good feeding practices. Botulism cannot be directly transmitted from diseased animals to the humans who handle them. But potential sources of contamination are feces, tissues, and body fluids from animals with botulism, which should be handled with care and marked with warning labels before they are sent to the laboratory, because of the potency of the toxin.

Several outbreaks of botulism in human populations have been caused by contaminated products from commercial food companies. Failure in one step during food processing can be disastrous, as the potentially toxic product may rapidly be distributed worldwide. Botulism can be caused from unsuccessful heating, failure of pack integrity, or storage at temperatures allowing spore germination, multiplication and neurotoxin production. Outbreaks of botulism are also typically the result of preparation of traditional fermented foods that are eaten without heating. Thoroughly information about the risk factors will contribute to efforts to sharpen prevention and control strategies.

# 6 Sumár

Botulizmus je zriedkavé, ale vážne paralyticky prebiehajúce ochorenie spôsobené nervovým toxínom, ktorý je produkovaný sporulujúcou anaeróbnou baktériou *Clostridium botulinum*. Botulotoxín je jeden z najznámejších toxínov. Toxíny sa viažu na nervové zakončenia, kde na motorických neurónoch blokujú uvoľňovanie acetylcholínu, čo vedie k vzniku progresívnej paralýzy v organizme ľudí a zvierat. Bez rýchlej liečby s najväčšou pravdepodobnosťou dôjde k smrti v dôsledku ochrnutia dýchacích svalov.

Aj keď spóry *C. botulinum* sa bežne vyskytujú v životnom prostredí, predovšetkým v pôde, sedimentoch a vodách, vyklíčiť a rásť môžu iba za určitých anaeróbnych podmienok. K značným ekonomickým stratám dochádza nielen pri sporadickom, ale hlavne hromadnom výskyte nákazy, keď je často postihutý celý chov vďaka spoločnému prameňu nákazy (kontaminácia krmiva). V oblastiach kde je zvýšená prevalencia botulizmu, zvieratá (vrátane koní, hovädzieho dobytka, oviec, kôz, noriek a vtákov) sú pravidelne vakcínované.

Botulizmus u ľudí je zvyčajne spôsobený nesprávne konzervovanými potravinami. Alimentárny spôsob je najčastejším typom botulizmu u ľudí. Intoxikácie často vznikajú v dôsledku porúch v procese spracovania potravín. Ak je jedlo nedostatočne tepelne opracované, spóry dlhodobo prežívajú a produkujú toxíny. Medzi ďalšie faktory ovplyvňujúce prežívanie bakteriálnych spór v potravinárskom priemysle sú pH, zloženie vody, prostredie a konzervačné látky.

Výskyt botulizmu je vždy veľmi vážny medicínsky a veterinársky problém, ktorý je nutné okamžite riešiť. Rýchle rozpoznanie klinických príznakov a detekcia možného prameňa kontaminácie má zásadný vplyv pri zdolávaní botulizmu.

# 7 References


1. AKAKPO, A.J. – B. BONFOH. Botulism *In:* P. CH. Lefévre, J. Blancou, R. Chermette, G. Uilenberg (eds.): *Infectious and Parasitic Diseases of Livestock,* Lavoisier, Paris, 2010, ISBN: 978-2-7430-0872-7, p. 1221-1235.

2. ANDERSON, T. J. *et al*. Botulinum toxin treatment of spasmodic torticollis. In *Journal of the royal society of medicine*. [online]. 1992, vol. 85, no. 9 [cit. 2014-02-03]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1293637/>. ISSN 1758-1095

3. ANGULO, F. J. *et al*. A large outbreak of botulism: the hazardous baked potato. *In The Journal of infectious diseases*. [online]. 1998, vol. 178, no. 1 [cit. 2014-14-02]. Available on the Internet: <http://jid.oxfordjournals.org/content/178/1/172.long>. ISSN 00221899

4. ARNON, S. S. *et al*. Botulinum toxin as a biological weapon medical and public health management. In *The journal of the American medical association*. [online] 2001, vol. 285, no. 8 [cit. 2014-02-02]. Available on the Internet: <http://www.bt.cdc.gov/agent/botulism/botulismconsensus.pdf>. ISSN 1538-3598

5. BROWN, N. – DESAI, S. Infantile botulism: A case report and review. In *The Journal of Emergency Medicine*. [online]. 2013, vol. 15, no. 6 [cit. 2013-03-12]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/23871478>. ISSN 0736-4679

6. CDC (Centers For Disease Control and Prevention). Investigation of Anthrax Associated with Intentional Exposure and Interim Public Health Guidelines, October 2001. In *Morbidity and mortality weekly report*. [online] 2001, vol. 50, no. 41[cit. 2014-02-02]. Available on the Internet: <http://www.cdc.gov/mmwr/PDF/wk/mm5041.pdf>. ISSN 1545-861X

7. CFSPH (The Center for Food Security and Public Health). Botulism. [online]. 2010. [cit. 2013-07-12].
<http://www.cfsph.iastate.edu/Factsheets/pdfs/botulism.pdf>.

8. CHERINGTON, M. Clinical spectrum of botulism. In *Muscle & nerve*. [online]. 1998, vol. 21, no. 6 [cit. 2014-02-02]. Available on the Internet:
< http://www.ncbi.nlm.nih.gov/pubmed/9585323 >. ISSN 1097-4598

9. CIFUENTES-DIAZ, C. – FRUGIER, T. – MELKI, J. Spinal muscular atrophy. In *Seminars in Pediatric Neurology*. [online]. 2002, vol. 9, no. 2 [cit. 2014-02-03]. Available on the Internet:
<http://www.sciencedirect.com/science/article/pii/S1071909102800079>.
ISSN 1071-9091

10. COLEMAN, E. A. – YERGLER, M. E. Emergency: Mass Casualty: Botulism. In *The American Journal of Nursing*. [online]. 2002, vol. 102, no. 9 [cit. 2013-03-12]. Available on the Internet:
<http://ezproxy.uin.no:2075/stable/3522939?seq=1>. ISSN 0002936X

11. CONTI-FINE, B. M. *et al*. Myasthenia gravis: past, present, and future. In *The Journal of Clinical Investigation*. [online]. 2006, vol. 116, no. 11 [cit. 2014-02-03] Available on the Internet:
<http://www.jci.org/articles/view/29894>. ISSN 1558-8238

12. DAVIS, J. B. – MATTMAN, L. H. – WILEY, M. *Clostridium botulinum* in a fatal wound infection. In *Journal of the American Medical Association*. . [online]. 1951, vol. 146, no. 7 [cit. 2013-03-12]. Available on the Internet:
<http://www.ncbi.nlm.nih.gov/pubmed/14832034>. ISSN 0002-9955

13. DOELLGAST, G. J. *et al*. Enzyme-linked immunosorbent assay-enzyme-linked coagulation assay for detection of antibodies to Clostridium botulinum neurotoxins A, B, and E and solution-phase complexes. In *Journal of Clinical Microbiology*. [online]. 1994, vol. 32, no. 3 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC263141/pdf/jcm00003-0281.pdf>. ISSN 1098-660X

14. DRESSLER, D. Clinical applications of botulinum toxin. In *Current opinion in microbiology*. [online] 2012, vol. 15, no. 3 [cit. 2013-04-12]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S1369527412000665>. ISSN 1369-5274

15. EFSA (The European Food Safety Authority). *Opinion of the scientific panel on biological hazards on the request from the commission related to clostridium spp. in foodstuffs*. [online]. 2005. [cit. 2013-07-12]. <http://www.efsa.europa.eu/en/efsajournal/doc/199.pdf>

16. ERIKSEN, T. *et al.* Botulism infection after eating fish in Norway and Germany: two outbreak reports. In *Eurosurveillance*. [online]. 2004, vol. 8, no. 3 [cit. 2014-05-02]. Available on the Internet: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2366>. ISSN 1560-7917

17. FERNÁNDEZ, R. A. – CICCARELLI, A. S. Botulism: Laboratory Methods and Epidemiology. In *Anaerobe*. [online]. 1999, vol. 5, no. 3-4 [cit. 2014-02-02]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S1075996499902263>. ISSN 1075-9964

18. FOLKEHELSEINSTITUTTET (Norwegian Institute of Public Health). Botulisme. [online]. 2013. [cit. 2013-07-12]. <http://www.fhi.no/eway/default.aspx?pid=239&trg=Content_6493&Main_6157=6287:0:25,5499&MainContent_6287=6493:0:25,6833&Content_6493=6441:82659::0:6446:12:::0:0>.

19. GOULD, G. W. *New Methods of Food preservation*. New york: Aspen Publishers, 1995. 324 p. ISBN 0-8342-1341-9

20. GRANUM, P. E. Matforgiftning; næringsmiddelbårne infeksjoner og intoksikasjoner. Third edition. Norway: Høyskole-Forlaget AS, 2007. 406 p. ISBN 9788276346251

21. GRATTAN-SMITH, P. J. *et al*. Clinical and neurophysiological features of tick paralysis. In *Brain - A journal of neurology*. [online]. 1997, vol. 20, no. 11 [cit. 2014-02-03]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/9397015>. ISSN 1460-2156

22. GYLES, C. L.*et al*. Pathogenesis of Bacterial Infections in Animals. Fourth edition. Iowa, USA: 2010. 664 p. ISBN 978-0-8138-1237-3

23. HARDY, S. P. – KALDHUSDAL, M. Type C and C/D toxigenic Clostridium botulinum is not normally present in the intestine of healthy broilers. In *Veterinary microbiology*. ISSN 0378-1135, 2013, vol. 165, no. 3-4, p. 466-8.

24. HATHEWAY, C. L. Toxigenic clostridia. In *Clinical microbiology reviews*. [online] 1990, vol. 3, no. 1 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC358141/>. ISSN 098-6618

25. HAUSCHILD, A.H.W - DODDS, K. L. *Clostridium botulinum: Ecology and control in foods*. New york: Marcel Dekker, 1993. 424 p. ISBN 0-8247-8748-X

26. KAHN, C. M. *The Merck Veterinary Manual*. Tenth edition. New Jersey, USA: Merck & Co., Inc 2010, 2945 p. ISBN 0-911910-93-X

27. KARALEWITZ, A. P.-A. – BARBIERI, J. T. Vaccines against botulism. In *Current opinion in microbiology*. [online] 2012, vol. 15, no. 3 [cit. 2014-02-02]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S0264410X09012134?via=ihub> ISSN 1879-0364

28. KOTHARE, S. V. - KASSNER, E.G. Infant botulism: a rare cause of colonic ileus. In *Padiatric radiology*. [online]. 1995, vol. 25, no. 1 [cit. 2014-05-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/7761156>. ISSN 1432-1998

29. KUMAR, P. – CLARK, M. Clinical medicine. Third edition. London: W. B. Saunders Company, 1994. 1135 p. ISBN 0-7020-1739-6

30. LIGUORI, V. *et al*. A Case of Wound Botulism in a Foal Affected by Gastric Ulcers in Italy. In *Journal of Equine Veterinary Science*. [online] 2008, vol. 28, no. 8 [cit. 2014-02-02]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S0737080608002050>. ISSN 0737-0806

31. LINDSTRØM, M. *et al.* Type C Botulism Due to Toxic Feed Affecting 52,000 Farmed Foxes and Minks in Finland. In *Journal of Clinical Microbiology*. [online] 2004, vol. 42, no. 10 [cit. 2014-02-02]. Available on the Internet: <http://jcm.asm.org/content/42/10/4718.full>. ISSN 1098-660X

32. LINDSTRØM, M. – KORKEALA, H. Laboratory Diagnostics of Botulism. In *Clinical Microbiology Reviews*. . [online]. 2006, vol. 19, no. 2 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1471988/>. ISSN 1098-6618

33. MACDONALD, E. *et al*. Outbreak of wound botulism in people who inject drugs, Norway, October to November 2013. In *Eurosurveillance*. [online]. 2013, vol., 18, no. 45 [cit. 2013-03-12]. Available on the Internet: <http://www.eurosurveillance.org/images/dynamic/EE/V18N45/art20630.pdf>. ISSN 1560-7917

34. MASELLI, R. A. – BAKSHI, N. Botulism. In *Muscle & Nerve*. [online]. 2000, vol. 23, no. 7 [cit. 2013-03-12]. Available on the Internet: <http://onlinelibrary.wiley.com/doi/10.1002/1097-4598(200007)23:7%3C1137::AID-MUS21%3E3.0.CO;2-7/abstract>. ISSN 1097-4598

35. MATTINGLY, E. [online].[Cit. 2013-10-03]. <http://www.physio-pedia.com/Botulism>.

36. MCDONALD, P. *et al. Animal Nutrition*. Seventh edition. Essex, England: Pearson, 2011. 692 p. ISBN 978-1-4082-0423-8

37. MCVEY, S. D. – KENNEDY, M. – CHENGAPPA, M. M. *Veterinary Microbiology*. Third edition. Iowa, USA: Wiley-Blackwell, 2013. 648 p. ISBN 978-0-470-95949-7

38. MYLLYKOSKI, J. *et al*. Fur animal botulism hazard due to feed. In *Research in veterinary science*. [online] 2011, vol. 90, no. 3 [cit. 2013-10-11]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S0034528810002316?via=ihub.> ISSN 1532-2661

39. NEWSON-DAVIS, J. Lambert-Eaton Myasthenic Syndrome. In *Revue Neurologique*. [online]. 2004, Vol. 160, no. 2 [cit. 2014-02-03]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S0035378704708887>. ISSN 0035-3787

40. NIAID (National Institute of Allergy and Infectious Diseases). Botulism. [online]. 2010. [cit. 2014-02-02]. <http://www.niaid.nih.gov/topics/botulism/Pages/default.aspx>.

41. OSTROWSKI, S. R. *et al.* An outbreak of equine botulism type A associated with feeding grass clippings. In *Journal of veterinary diagnostic investigation: official publication of the American Association of the Veterinary Laboratory Diagnosticians*. [online]. 2012, vol. 24, no. 3 [cit. 2014-02-02]. Available on the Internet: <http://ezproxy.uin.no:2408/content/24/3/601.full> ISSN 1040-6387

42. PATERSON, D. L. *et al*. Severe botulism after eating home-preserved asparagus. In *The Medical journal of Australia*. [online]. 1992, vol. 157, no. 4 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/1435446>. ISSN 1326-5377

43. POEWE, W. *et al*. Treatment of spasmodic torticollis with local injections of botulinum toxin. One-year follow-up in 37 patients. In *Journal of Neurology*. [online]. 1992, vol. 239, no. 1 [cit. 2014-02-03]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/1541964>. ISSN 1432-1459

44. ROSS, A. I. *et al*. Combining nonthermal technologies to control foodborne microorganisms. In *International Journal of Food Microbiology*. [online]. 2003, vol. 89, no. 2-3 [cit. 2014-05-02]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S0168160503001612>. ISSN 1879-3460

45. SCHIAVO, G. – MATTEOLI, M. – MONTECUCCO, C. Neurotoxins affecting neuroexocytosis. In *Physiological reviews*. [online] 2000, vol. 80, no. 2 [cit. 2013-04-12]. Available on the Internet: <http://physrev.physiology.org/content/80/2/717>. ISSN 1522-1210

46. SCHMIDT, R. D. – SCHMIDT, T. W. Infant botulism: A case series and review of the literature. In *The Journal of Emergency Medicine*. [online]. 1992, vol. 10, no. 6 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/1491154>. ISSN 0736-4679

47. SHAPIRO, R. L. – HATHEWAY, C. – SWERDLOW, D. L. Botulism in the United States: a clinical and epidemiologic review. In *Annals of internal medicine*. [online]. 1998, vol. 129, no. 3 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/9696731>. ISSN 00034819

48. SHARMA, S. K. – SINGH, B. R. Hemagglutinin binding mediated protection of botulinum neurotoxin from proteolysis. In *Journal of natural toxins*. [online].1998, vol. 7, no. 3 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/9783262>. ISSN 1058-8108

49. SIMPSON, L. L. Identification of the major steps in botulinum toxin action. In *Annual review of pharmacology and toxicology*. [online] 2004, vol. 44 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/14744243>. ISSN 1545-4304

50. SMITH, L. A. Botulism and vaccines for its prevention. In *Vaccine*. [online] 2009, vol. 5, no. 27 [cit. 2014-02-02]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S0264410X09012134?via=ihub> ISSN 1873-2518

51. SMITH, L. DS. *Botulism – The organism, Its toxins, The disease*. Springfield, Illinois, USA: Charles C Thomas Publisher, 1977. 236 p. ISBN 0-398-03543-1

52. SPIKA, J.S. *et al*. Risk factors for infant botulism in the United States. In *American journal of diseases of children*. [online]. 1989, vol. 143, no. 7 [cit. 2014-02-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/2741856>. ISSN 0002-922X

53. SOLOMON, H. M. – KAUTTER, D. A. Outgrowth and toxin production by Clostridium botulinum in bottled chopped garlic. In *Journal of food protection*. [online]. 1988, vol. 51, no. 11 [cit. 2014-14-02]. Available on the Internet: <http://agris.fao.org/agris-search/search.do?recordID=US8904286>. ISSN: 0362-028X

54. SVA (Statens Veterinärmedicinska Anstalt). Förgiftningar och foderhygien. [online]. 2014. [cit. 2013-07-12] <http://www.sva.se/sv/Djurhalsa1/Hast/Forgiftningar-och-foderhygien/?lid=34129>.

55. STRANDWITZ, P. [online]. 2008. [Cit. 2013-10-03]. <http://bioweb.uwlax.edu/bio203/s2008/strandwi_phil/archive.htm>.

56. STUDDERT, V. P. – GAY, C. C. – BLOOD, D. C. Saunders Comprehensive Veterinary Dictionary. Fourth edition. USA: Elsevier Saunders, 2012. 1344 p. ISBN 978-0-7020-4743-5

57. SYKES, J. E. *Canine and Feline Infectious Diseases*. St. Louis, Missouri: Elsevier/Saunders, 2014. 928 p. ISBN 978-1-4377-0795-3

58. THAJEB, T. *et al.* Botulism: a frequently forgotten old malady. In *International Journal of Gerontology*. [online]. 2007, vol.1, no. 3 [cit. 2013-03-12]. Available on the Internet: <http://www.sgecm.org.tw/db/Jour/2/200709/3.pdf>. ISSN 1873-9598

59. TOWNES, J. M. *et al*. An outbreak of type A botulism associated with a commercial cheese sauce. In *Annals of internal medicine*. [online]. 1996, vol. 125, no. 7 [cit. 2014-02-02]. Available on the Internet: <http://www.med.mcgill.ca/epidemiology/courses/EPIB591/Fall%202010/Class%206%20-%2017%20Sept/TownesFullText.pdf>. ISSN 1539-3704

60. WALKER, B. Beating botulism. In *Nursing*. [online]. 2004, vol. 34, no. 6 [cit. 2013-03-12]. Available on the Internet: <http://journals.lww.com/nursing/Citation/2004/06000/Beating_botulism.56.Aspx> ISSN 03604039

61. WHITLOCK, R. H. – BUCKLEY, C. A. Botulism. In *The Veterinary clinics of North America. Equine practice*. [online] 1997, vol. 13, no. 1 [cit. 2013-04-12]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/9106347>. ISSN 1558-4224

62. WHITLOCK, R. H. – MCADAMS, S. Equine Botulism. In *Clinical Techniques in Equine Practice*. [online]. 2006, vol. 5, no. 1 [cit. 2014-02-02]. Available on the Internet: <http://www.sciencedirect.com/science/article/pii/S1534751606000084>. ISSN 15347516

63. WICHTEL, J. J. – WHITLOCK, R. H. Botulism associated with feeding alfalfa hay to horses. In *Journal of the American Veterinary Medical Association*. [online] 1991, vol. 199, no. 4 [cit. 2014-22-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/1917659>. ISSN 1943-569X

64. WINER, J. B. Guillain Barré syndrome. In *Molecular Pathology*. [online]. 2001, vol. 54, no. 6 [cit. 2014-02-03]. Available on the Internet: <http://mp.bmj.com/content/54/6/381.long>. ISSN 1472-4154

65. WHO (World Health Organization) Botulism. [online]. 2013. [Cit. 2013-07-12] <http://www.who.int/mediacentre/factsheets/fs270/en/>.

66. YNDESTAD, M. - HELGEBOSTAD, A. - LOFTSGÅRD G. Susceptibility of foxes to Clostridium botulinum type C and E toxins. In *Acta Veterinaria Scandinavica*. [online]. 1977, vol. 18, no. 1 [cit. 2014-05-02]. Available on the Internet: <http://www.ncbi.nlm.nih.gov/pubmed/848403>. ISSN 1751-0147

67. ZIMMERMAN, J. J. *et al*. *Diseases of swine*. Tenth edition. UK: Wiley-Blackwell, 2012. 1008 p. ISBN 978-0-8138-2267-9