JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

NORWEGIAN FERMENTED FISH AS POSSIBLE SOURCE OF FOODBORNE BOTULISM – IS THE RISK OF CONTRACTING BOTULISM FROM FERMENTED FISH STILL RELEVANT?

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https://doi.org/10.55251/jmbfs.10116

ARTICLE INFO

Received 7. 2. 2023
Revised 2. 5. 2023
Accepted 9. 5. 2023
Published 1. 6. 2023

Regular article

OPEN ACCESS

ABSTRACT

Foodborne illnesses represent a substantial cause for disease among the population. The Norwegian fermented fish is a traditional way of conserving fish during winter. If wrongly prepared or stored, fish contaminated with *Clostridium botulinum* may be contaminated with neurotoxin due to favorable conditions for the anaerobic bacterium. The classical form of botulism is due to ingestion of preformed neurotoxin in food that has been contaminated with spores. Hygiene is one of the main preventive factors in order to decrease contamination of the fish with unwanted bacteria, including *C. botulinum*. Norwegian fermented fish („rakfisk“) is made with attention to key steps and strategies to prevent contamination by bacteria, with *C. botulinum* especially. The temperature and salt concentration are some of the most important hurdles in order to prevent unwanted microbial growth. The hurdle principle refers to a combination of obstacles which are placed to have a safe end-product. Botulism is a rare disease in Norway, but it does occur. As such it is still relevant when discussing „rakfisk“, especially if it is homemade. Clinicians should be aware of botulism and „rakfisk“ could provide an important diagnostic clue when the condition is suspected.

Keywords: foodborne illnesses; botulism; *Clostridium botulinum*; contamination; fermented fish

INTRODUCTION

Foodborne diseases are very common throughout the world with causative agents greatly varying between countries, continents and socioeconomic groups. In developing countries gastrointestinal conditions can be potentially life-threatening due to associated dehydration, especially in children **(WHO, 2015)**. In 2015 the World Health Organization published the report “WHO estimates of the global burden of foodborne disease”, as the first of its kind. It is a large of scope, detailed report, including the incidence of 31 different bacteria, virus, parasites and chemicals as etiology of foodborne illnesses represented by country. Foodborne illnesses represent a substantial cause for disease among the population. It is noticeable that the associated mortality rate to the disease is low in the European regions **(Zuraw, 2015)**.
The Norwegian fermented fish is a traditional way of conserving fish during winter. If wrongly prepared or stored, fish contaminated with *Clostridium botulinum* may be contaminated with neurotoxin due to favorable conditions for the anaerobic bacterium. The goal of this article is to assess where the risk for contamination is high during the process of preparing the fish, and what conditions needed in order for the neurotoxin to be formed. In addition, attention is paid to whether the risk of contracting botulism from ingestion of Norwegian fermented fish is still relevant nowadays.

CLOSTRIDIUM BOTULINUM AND BOTULISM – KEY FACTS

C. botulinum is a common finding in the environment and can be found worldwide. It is for example present in seafood, soil, sea water, marine sediment and the gastrointestinal tract of healthy fish **(Müller *et al.*, 2010; WHO, 2016)**. Some strains are also known to colonize the gastrointestinal tract in humans, fish and other animals **(Espelund and Klaveness, 2014)**.
C. botulinum is a gram positive, rod shaped, strict anaerobic bacterium which is capable of forming very resistant endospores. In the absence of oxygen, they germinate and start to grow and form exotoxin. The size varies from 1-30 micrometer, making it a relatively large bacterium. The vegetative form of the bacterium produces the exotoxin - botulinum toxin - which is released during cell growth and lysis. The name botulinum is derived from latin (bolus means sausage) due to an event of “sausage poisoning” (4) in a southern town in Germany in the 1820 **(Kerner, 1982)**. It produces the most potent neurotoxin known and it causes the disease botulism. The disease is most often due to ingestion of preformed neurotoxin by the bacterium **(Müller *et al.*, 2010; Espelund and Klaveness, 2014)**. Eight serologically different types of neurotoxins have been described, from A - H. H being the latest addition, described in 2014 **(Dover *et al.*, 2014)**. Neurotoxin type A, B and E are the commonest types seen with disease in humans. As a general rule one strain of bacteria “almost always produces only one toxin type”. Botulinum neurotoxin is the most potent toxin known, and it is estimated that one gram of aerosolized toxin has the capability of killing 1,5 million people (WHO, 2016). The classical form of botulism is due to ingestion of preformed neurotoxin in food that has been contaminated with spores and not adequately heated, one example being fermented fish **(Müller *et al.*, 2010)**.

Diagnosis of foodborne botulism

Diagnosis of foodborne botulism is made by proving the presence of *C. botulism* in suspected food and in feces. Demonstration of neurotoxin in above mentioned material as well as serum should also be done. There are several ways of laboratory confirmation of the presence of toxin. One method is the confirmation of neurotoxin in blood or feces **(Farbu *et al.*, 2015)**. This method of investigation is very sensitive, however, it is expensive and have ethical dilemmas, since the end-result of the trial is death of an animal **(Lindström and Korkeala, 2006)**. Enzyme-linked Immunosorbent Assay (ELISA) is an example of an immunological method where botulism neurotoxin is detected. Endopeptidase assays exploits the fact that botulinum neurotoxin are zinc endopeptidases, cleaving specific proteins in the synaptic cleft, including – Soluble N-ethylmaleimide attachment protein receptor (SNARE). In combination with immunological detection of the cleaved peptide or detection of fluorescence when a labeled peptide is cleaved this provide a highly specific way to detect the biological active neurotoxins **(Asensio *et al.*, 2008)**.
Determination of neurotoxin - in special laboratories - proof - fish sample, biological sample from a patient (stomach contents), rarely determined or proven, because it is unstable and proof of botulinum toxin from a phoretic point of view cannot be done very often.
Identification of neurotoxin by MALDI - is difficult, determination of C. botulinum, identification of individual proteins and botulinum toxin is only produced in food,

Enzyme-Linked Immunosorbent Assay

Direct diagnosis - proof of neurotoxin and and proof of antibodies (neutralization tests) are carried out in a highly specialized workplace (phoresis).

Enzyme-linked immunosorbent assay (ELISA) is a biochemical procedure that utilises antibody conjugated enzymes to detect the presence of a specific antigen **(Asensio *et al.*, 2008)**. There are three common set ups: direct, indirect and sandwich (capture), as shown in Figure 1. Image of common ELISA set ups: Direct, Indirect and Sandwich. In both direct and indirect ELISA, antigens (Ag) are bound to the microtiter plate first, an antibody specific to the antigen is then introduced. In direct assays this primary antibody (blue) has been modified with an enzyme (red star) such as HRP, which when exposed to a substrate produces a measurable colour change. In indirect ELISA, this enzyme is bound to a secondary antibody (green) that has been modified with an enzyme to facilitate colour change; this secondary antibody binds to the primary antibody. In sandwich ELISA, the surface is treated with a capture antibody (red) specific to a desired antigen before the rest of the assay proceeds in the same manner as the indirect assay.

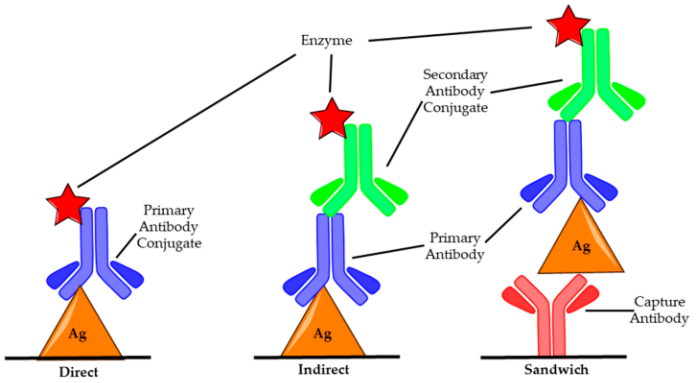

Figure 1 Common set ups: Direct, undirect, and sandwich.

For direct and indirect ELISA, the sample to be tested is first applied to the microtiter plate, allowing any antigens present to bind before blocking solution, commonly bovine serum albumin (BSA) or casein, is added in order to prevent any non-specific binding of antibodies **(Eteshola and Leckband, 2001)**. Next, the primary antibody that is specific to the antigen is added and allowed to bind. In the case of direct ELISA, this antibody is conjugated to an enzyme which on addition of its substrate produces a measurable colour change signal. With indirect ELISA, a secondary antibody which contains the enzyme conjugate is used to produce the response. The detection of BoNT complexes in immunological assays is hampered by the presence of neurotoxin-associated proteins (NAPs). It is this NAP complex that blocks antigenic sites of the toxin, making them unavailable for binding to antibodies, meaning many assays are developed using highly purified BoNT samples, which is not advantageous for use in diagnostic and food-testing **(Szílagyi *et al.*, 2000)**.

Biosensors

Biosensor technologies embody a wide and diverse range of BoNT detection methods, usually, the base platforms used consist of: surface plasmon resonance (SPR), refractometer, fluorescence and chemical luminescence. Typically, evanescence wave technology is used for fluorescence-based biosensors. Molecules that are labelled with fluorophores and bound to a surface become excited upon exposure to evanescent fields, resulting in the production of a signal. Often the assay used is an immunological sandwich assay, consisting of two antibodies for immobilisation and quantification along with the analyte of interest, all of which subsequently locate on the sensor surface. Utilising this system, the sensitivities of sensors tested ranged from as little as 150 pg/mL **(Singh and Silvia, 1996)** to 200 ng/mL **(Kalb *et al.*, 2008)** for botulinum neurotoxin serotypes E and B, respectively. Generally, biosensor-based platforms take >20 minutes to complete, with multiple analytes detectable simultaneously, depending on the sensor design, this would be of particular interest in development of the system for its use in the diagnostic and food testing sectors. The speed with which results can be obtained make biosensors one of the most rapid available platforms around. Evidence of increased sensitivity is demonstrated by the recently developed Newton Photonics SPR biosensor, which has a LoD quantified at 6.76 pg/mL (BoNT/A light chain), allowing for active toxin quantification, which is advantageous for its adoption in the pharmaceutical production sector. The SPR method has a detection time of less than 20 minutes.

Endopeptidase Mass Spectrometry

Endopeptidase Mass Spectrometry (Endopep-MS) is modern technique assay which require the use of serotype-specific antibodies, see Figure 2 for diagrammatic overview. The antibodies are conjugated to magnetic beads and then added to a sample (a); the beads are removed and thoroughly washed before a substrate that imitates the toxins natural target is added (b). The solution is then incubated, and the resulting mixture is analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) **(Singh and Silvia, 1996)**. The MS detects any whole substrate and any cleaved fragments that result from incubation with the active toxin (c) **(Kalb *et al.*, 2008)**. Determination of neurotoxin - in special laboratories - proof - fish sample, biological sample from a patient (stomach contents), rarely determined or proven, because it is unstable and proof of botulinum toxin from a phoretic point of view cannot be done very often.

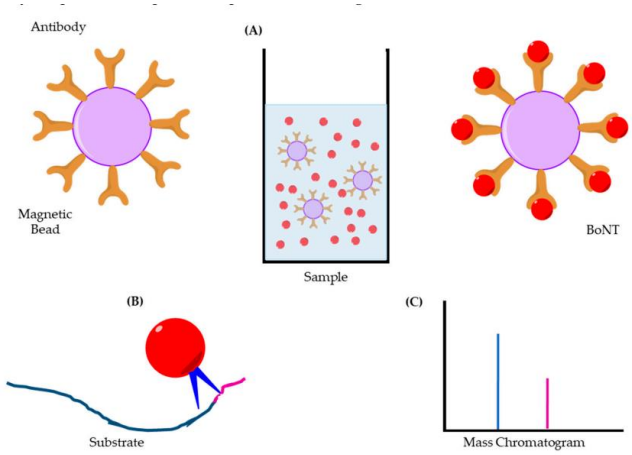

Figure 2 An overview of the endopeptidase mass spectrometry assay

The procedure takes around 4–8 h. Rosen *et al.* have developed a multiplex platform to allow the assay to simultaneously detect BoNT/A, B and E. The sensitivity achieved is good with a detectable range across 100 fg/mL to 1 ng/mL, depending on the sample matrix. The wide range of sample matrices tested, including serum, stool, culture supernatants, and a diverse set of food samples again reinforces its potentially extensive application.

Prophylaxis of botulism

Procedures in the production and storage of fish help to prevent the occurrence of C. botulinum infection. A previous infection with *C. botulinum* does not render the person immune to a subsequent infection with the bacterium **(Kalb *et al.*, 2006)**. This is illustrated by a case report from Bilusic et all from 2008 where a patient presented with botulism in 2006 and 2007 from eating by the same jar of contaminated hot chili peppers **(Harvey *et al.*, 2013)**. The fundamental prophylaxis of botulism poisoning is proper handling and preparing of food, with close attention to canning (especially at home in a non-professional environment), avoiding contamination and to construct a non-beneficial environment facilitating neurotoxin formation by spores. Several of these factors are addressed individually in the fermenting process of the Norwegian specialty – „rakfisk“ **(Müller *et al.*, 2010)**. Foodborne botulism can be prevented by using proper food canning techniques. The best home remedy to ensure germ-free food processing is to pressure-cook the food at 121°C for 20 – 100 minutes, depending on the food item. Before serving, the food should be boiled for at least 10 minutes. For proper storage of canned foods, it is important to follow the manufacturer's instructions. Ensure storing the cold food below 5°C and hot food above 60°C. Low acidic foods, such as asparagus, green beans, beets, corn, and potatoes, are potential sources of foodborne botulism **(Stone, 2017)**. The risk of botulism can also be reduced by refrigerating the canned products after opening the seal. To avoid wound botulism, properly clean the wound. To avoid infant botulism, do not give honey to infants under the age of 1 year **(Barash and Amon, 2014)**.

NORWEGIAN FERMENTED FISH – „RAKFISK“

The first written source referring to „rakfisk“ in Norway dates to 1348. The word "rak" have long traditions. Ivar Aasen, a Norwegian philologist and lexicographer, wrote in 1848 that "rak" refers to the specific treatment of fish resulting in a soft, moist texture and sour taste. The Icelandic word "rakur" refer to something moist as well as the word "rakr" from the etymological dictionary "Nynorsk etymologisk ordbok", written by Alf Torp in 1918. A. Torp adds in his definition the latin word "rigare" which has a meaning related to water. The process of fermenting in Norway is traditionally referred to as "rake", hence the descriptive term "rakfisk" **(Bilusic *et al.*, 2008)**. Traditionally, fermentation of fish had its natural implication as a food conserving method. In order to have enough food during winter, fermentation had its natural place since fresh fish could be preserved and eaten months later. The most common fish to be used for the fermentation process nowadays are the trout (Salomon trutta L.), but the common whitefish and charr may also be used **(Berg, 2009)**.

PREPARING OF NORWEGIAN FERMENTED FISH – „RAKFISK“

Hygiene is one of the main preventive factors in order to decrease contamination of the fish with unwanted bacteria, including *C. botulinum*. For instance, fish in

contact with the ground or soil for any reason are at a higher risk of *C. botulinum* contamination and subsequent development of neurotoxin in the finished product. Therefore, strict measures should be taken before attempting the process of fermentation **(Lines and Spence, 2014)**. The fish farm employees wear dresses and gloves to minimize the risk. Included is also good routines for hand washing, cleaning of all surfaces related to fish production, filtering of water and air, keeping the air inside the locals at a positive pressure and ultraviolet lights. In these ways the production local has some parallels to a surgical operating theatre. Norwegian fermented fish („rakfisk“) is made with attention to key steps and strategies to prevent contamination by bacteria, with *C. botulinum* especially in mind. The process by which „rakfisk“ is made can be divided into four general steps **(Jobling, 1981)**.

First step of processing procedure

Before the fish enter the production via a pipeline from the breeding pond, it has fasted for the duration of approximately one week. This aims at reducing the gastrointestinal content and fecal contamination **(Skåra *et al.*, 2015)**. The buildup of carbon dioxide and ammonia in the water during crowding of the fish prior to slaughter is also less, as fish adapts their metabolic rate to the decreased food intake. Post-prandial oxygen consumption increases, in relation to the increased absorption of nutrients from the alimentary tract, and an increased excretion of ammonia **(Schmidt-Nilsen and Bøhmer, 1935)**. Due to a natural difference in height between the breeding pond and the location of the production unit, the water is moving according to the forces of gravitation. In the production unit, the fish enters an apparatus delivering an electrical current. This method is approved by the Norwegian Food Safety Authority as a humane method of anesthetizing/killing of the fish. At fish farm the electrical stunning is done with the fish out of water (dry stunning), one shock is provided when the fish is in contact with the belt and the panel above. This type of stunning is rarely permanent, and for fish to regain consciousness depends upon further management. Once the fish has been stunned, the main artery is cut below the head and the fish placed in a tub with still water in order to bleed out **(Kjos-Hansen, 1986)**.

Figure 3 In the tub the fish bleeds out and the full length of the abdomen is cut open before placed onto the slide seen on the right in order to be further processed. Photo: Henrik A. Kildahl with courtesy from Noraker farm.

Second step of processing procedure

Removal of the abdominal content is important to manage with care in order to prevent perforation. As mentioned, *C. botulinum* may be found in the gastrointestinal tract of healthy fish, and thus, perforation of the gut poses a possible source of contamination. At visited farm the abdominal content is removed by means of a vacuum system. A pipe with continuous suction takes the gastrointestinal content of the fish into a tank and it ultimately ends up as fertilizer. With a gliding hand motion, the fish is rubbed against the sharp bottom end of the pipe to remove the alimentary organs **(Singh and Silvia, 1996; Axelsson, 2008)**.

Third step of processing procedure

The fish is washed under high pressured water in order to remove contaminating materials (Figure 4.). Manual force may be used, but brushes and other equipment is avoided for hygienic reasons. The fish is subsequently sorted and those with defects such as a broken skin, deformities or other defects are sorted out. The fish is then weighed and put into buckets of predetermined sizes and weights (3, 5, 10 kilograms). The amount of salt added is calculated from the total weight of fish in a bucket. In a bucket with 10 kilogram fish, 420 grams salt is added in order to reach a concentration of 4,2 %. Most producers of rakfisk have a salt concentration between 4-6 %. Salt is placed within the fish as well as between the fish layers in the bucket and placed in cold storage at 7 °C. The amount of 40 time used from the fish swims up from the pipe until it is placed in a bucket in cold storage should be less than two hours **(FHI, 2016)**.

Figure 4 Washing station with filtered water under high pressure. Photo: Henrik A. Kildahl with courtesy from Noraker farm.

Fourth step of processing procedure

During first two days in cold storage, brine is created from the addition of salt to the fish via osmosis. The buckets are then opened, if the fish is not completely covered with brine, more is added, to completely cover the fish to prevent the presence of oxygen and secure an anaerobic environment within the bucket. The buckets are then stored for a variable period of time, until the fermentation process is complete (Landsverk, 2013).

Cold storage

The duration of time the fish is placed in cold storage depends upon the temperature and salt concentration. At this particularly farm the time varies between 4 – 12 months. The duration of fermentation is reflected in the taste of the fish, thus three principal types of rakfisk is classified according to taste and duration of storage **(Kalb *et al.*, 2006)**:

Table 1 Relation between duration of storage of rakfisk and strength of taste **(Kalb *et al.*, 2006)**

4-7 months	Mild
7-12 months	Stored
More than 12 months	Well-stored

Hurdle principle

Traditionally Norwegian fermented fish was prepared according to the “rule of 6s”. 6% salt, 6 °C for 6 weeks. The temperature and salt concentration are some of the most important hurdles in order to prevent unwanted microbial growth. The hurdle principle refers to a combination of obstacles which are placed to have a safe end-product.

Table 2 Important hurdles used in the process of fermentation **(Kalb *et al.*, 2006)**

- Temperature
- Salt concentration
- Acidic pH
- Function of the lactobacillus
- Container material

Environmental parameters that stimulate spores of *C. botulinum* to germinate and produce botulism neurotoxin, include **(WHO, 2016)**:

• pH > 4,6
• Temperatures between 25 – 37 C°
• Anaerobic environment

Many of the hurdles in the table above aims to minimalize these parameters. The temperature during fermentation is kept between 3 – 7 C° at all the time. The salt concentration varies between the producers of „rakfisk“, but is generally between 4 – 6% (Sobel, 2004), **(Fleck-Derderian *et al.*, 2018)**.

Fermentation process

It has been determined that the process causing the maturation of rakfisk is a combination of an autolytic process due to proteolytic enzymes present in the fish and activity of microbiological organisms, especially lactic acid producing bacteria

(Harvey *et al.*, 2013). The most dominant bacterium during a standard fermentation process of rakfisk is lactobacillus **(Scalfaro *et al.*, 2019; Mezencev and Klement, 2017)**, with the most common serotype being *L. sakei* **(Chen *et al.*, 2021; De Medici *et al.*, 2009**. During the ripening of rakfisk there were cultivated non-pathogenic gram-negative bacteria at concentrations of 10 000/gram as well as low presence of yeasts **(Chen *et al.*, 2021)**.

EPIDEMIOLOGY OF BOTULISM IN NORWAY

According to the Norwegian Surveillance System of Communicable Diseases (MSIS), the incidence of botulism, in total, from 1977 – 2017 is presented in Figure 5.

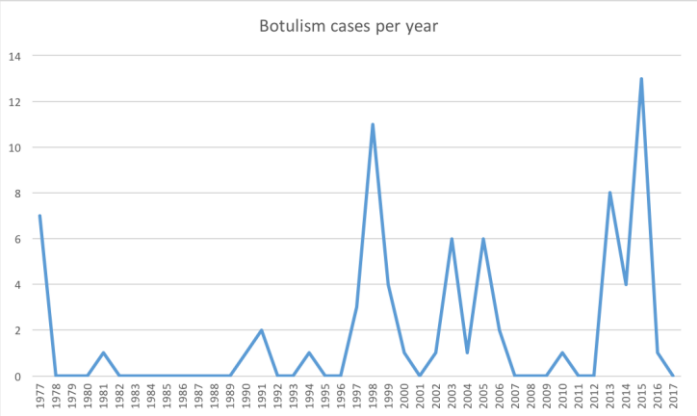

Figure 5 Norwegian Surveillance System of Communicable Diseases, Norwegian Institute of Public Health on 19.1. 2017.

According to MSIS there may be irregular delays in the reporting of data, providing another potential reason to why the data may not always reflect the true numbers of any given period. It is also important to note that these are the total numbers of botulism cases registered, it does not differentiate between foodborne, infant or wound botulism. According to a report conducted by the Norwegian Food Safety Authority regarding supervision of producers/refiners of small-scale freshwater fish, the total number of registered cases of foodborne botulism in the period 1977-2015 was 38 of a total number of 73 cases (Figure 6) **(Lines and Spence, 2014; Lonati *et al.*, 2020)**. When the traditional Norwegian „rakfisk“ (special method of fermenting fish) were implied, it was due to inadequate standards in a non-professional environment. Due to possible presence of *C. botulinum* in the gastrointestinal flora of fish as well as no practical options for controlling the finished product, control of the production, hygiene, temperature, and salt concentration is of prime importance **(Lonati *et al.*, 2017; Rossetto *et al.*, 2019)**. The type of neurotoxin that causes botulism in humans varies geographically. In Norway the most common toxin type is E, while B is the most common in central Europe. In the United States A is more common, even though E is prevalent as well. The reason is high numbers in Alaska due to ingestion of traditionally prepared food, including fermented fish. These variations can be explained by the prevalence of different types of neurotoxins associated with different foods **(Aureli *et al.*, 1986; Newkirk and Hedberg, 2012)**.

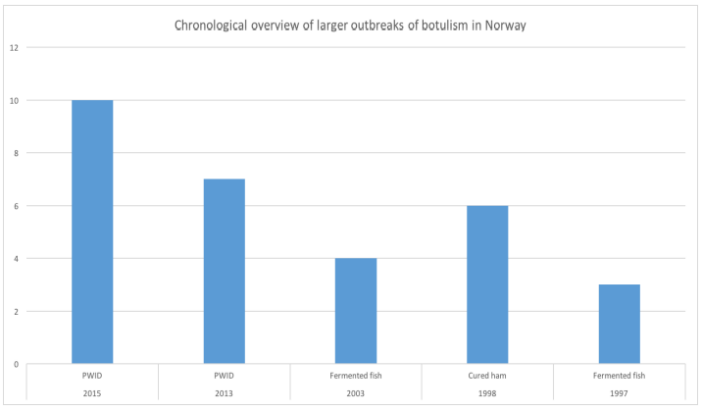

Figure 6 Overview of larger outbreaks of botulism in Norway, based upon etiology. PWID = People Who Inject Drugs, in these cases heroin was implied. The fermented fish in question was homemade under less-than –favorable conditions **(Lines *et al.*, 2014)**.

CONCLUSIONS

Norwegian fermented fish is a traditional way of preparing different types of fish in as a way of conserving the food. Botulism is a lethal condition which may result in death without treatment. It is possible to develop the condition after ingestion of inadequately prepared rakfisk favoring the neurotoxin formation from *C.botulinum*. Since 1977, 38 cases of foodborne botulism have been reported to the Norwegian Food Safety Authority. Most of the recent cases of botulism are attributed to drug abuse with heroin. In depth knowledge about the fermentation process and the potential hazards of wrong storage has resulted in the development of the hurdle principle which effectively put several barriers in place. The hurdle principle effectively prevents botulinum toxin, as well as potential harmful bacterial flora, from being developed within the stored fish. However, strict guidelines and controls from the Norwegian Food Safety Authority have resulted in an improvement of knowledge and generally better quality of the fermentative process. The cases which have been reported are invariable due to homemade rakfisk where the end product have been contaminated with toxin-forming *Clostridium botulinum* bacteria and conditions allowing formation of neurotoxin. Botulism is a rare disease in Norway, but it does occur. As such it is still relevant when discussing „rakfisk“, especially if it is homemade. Clinicians should be aware of botulism and „rakfisk“ could provide an important diagnostic clue when the condition is suspected.

Conflicts of interest: The authors declare that no conflicts of interest exist.

Ethical approval: Ethical approval is not needed for this article.

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