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The case of botulinum toxin in milk – experimental data

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Abstract

Botulinum neurotoxin (BoNT) is the most toxic substance known to man and the causative agent of botulism. Due to its high toxicity and the availability of the producing organism *Clostridium botulinum*, BoNT is regarded as a potential biological warfare agent. Because of the mild pasteurization process, as well as rapid product distribution and consumption, the milk supply chain has long been considered a potential target of a bioterrorist attack. Since no empirical data on the inactivation of BoNT in milk during pasteurization, to our knowledge, are available at the present time, we investigated the activity of BoNT/A and BoNT/B as well as their respective complexes during a laboratory scale pasteurization process. When we monitored milk alkaline phosphatase activity, which is an industry accepted parameter of successfully completed pasteurization, our method proved comparable to the industrial process. After heating raw milk spiked with set amounts of BoNT/A, BoNT/B or their respective complexes, the structural integrity of the toxin was determined by ELISA and its functional activity by mouse bioassay. We demonstrated that standard pasteurization at 72°C for 15 seconds inactivates at least 99.99% of BoNT/A and BoNT/B, and at least 99.5% of their respective complexes. Our results suggest that if BoNT or their complexes were deliberately released into the milk supply chain, standard pasteurization conditions would reduce their activity much more dramatically than originally anticipated, and thus lower the threat level of the widely discussed "BoNT in milk" scenario.

Introduction

Botulinum neurotoxin (BoNT) is mainly produced by the rod-shaped anaerobic bacterium *Clostridium botulinum*, but can also be produced by unique strains of *C. baratii* and *C. butyricum* (15). The toxin is a di-chain protein with a molecular weight of 150 kDa, consisting of a heavy chain of 100 kDa and a light chain of 50 kDa which are linked by a disulfide bond. All known BoNT types are secreted bound to non-toxic neurotoxin associated proteins (NAP) and assemble into large complexes with molecular masses of 300, 600 or 900 kDa, depending on the toxin type (20, 23, 39). There are seven known antigenically distinct BoNT serotypes, designated as types A through G (11, 32). In recent years, BoNT serotypes have been further grouped into subtypes, e.g. A1-A5, differentiated on the basis of the variability of BoNT genes, deduced protein sequences and their immunological properties (18, 24, 28, 40). BoNT/A, /B, /E, and /F are the causative agents of foodborne botulism in man, a serious paralytic illness, which is the result of consuming improperly preserved food contaminated with *C. botulinum* spores and/or BoNT (16, 26, 31). After oral ingestion BoNT reaches the intestinal tract where the NAP associated with BoNT in the complex are believed to protect the toxin against digestive enzymes during its passage through low pH gastric juice (25). After crossing the intestinal mucosa, BoNT is circulated through the blood, ultimately reaching the neuromuscular nerve endings (22, 29). Specific binding of the heavy chain to receptors on the nerve cell surface triggers the translocation of BoNT into the lumen of the cell (3, 14, 34). Once in the cytosol of the nerve cell, the release of acetylcholine is inhibited by the endopeptidase activity of the BoNT light chain, leading to symmetric descending, flaccid paralysis (38). There is no reliable data on the exact oral toxicity of BoNT for humans. However, from animal studies using nonhuman primates and from cases of human botulism it is estimated that the lethal oral dose of BoNT is between 10 ng and 1 µg kg^{-1} body weight (17, 27). Generally, the lethal toxicity depends on the BoNT serotype and the route of exposure (2, 9), and it may vary across individuals.

Given its extreme toxicity, the Centers for Disease Control and Prevention (CDC, Atlanta, USA) lists BoNT as a Category A bioterrorism agent: high-priority agents and organisms that pose a high risk to public health and national security (7). Consequently, there were serious concerns that terrorists could attack the population by contaminating food staples with BoNT or a similar agent. The milk supply, in particular, was considered as a likely target since the many transportation and processing steps between cow and consumer represent critical and vulnerable points at which bioterrorism agents could be deliberately released. In addition, milk and other dairy products are distributed rapidly after packaging and, as one of the most widely consumed foodstuffs, reach large parts of the population.

In 2005, Wein and Liu described a theoretical scenario of a bioterror attack on the milk supply chain based on a series of mathematical calculations. It garnered considerable interest among the scientific community, politicians and intelligence organizations (41). Referring to experiments in which the toxic activity of BoNT in different foodstuffs, but not milk specifically, was determined after heat treatment (42), they based their calculation on roughly 70% thermal inactivation of BoNT during pasteurization, leading to 105 fatalities after the release of 1 g BoNT into raw milk prior to industrial pasteurization. As the scenario was based on theoretical assumptions, it seemed crucial to us to quantify the actual inactivation rate of BoNT in raw milk in a close-to-industry pasteurization process, as this would allow us to calculate a reliable scenario.

The heat treatment commonly used by the dairy industry is high temperature short time (HTST) pasteurization, a continuous process where a plate heat exchanger is used to rapidly bring the milk up to the required temperature of 72°C and hold it steady at this temperature for at least 15 seconds. Subsequently, the milk is cooled to 4°C and packaged for consumption. The pasteurization process is controlled by a standard method which measures the activity of milk alkaline phosphatase (ALP) as an intrinsic time-temperature integrator (1, 13, 21, 33). If HTST pasteurization is successful, the activity of ALP falls below 350 mU L^{-1}, the threshold set by the International Dairy Federation and the International Organization for Standardization (21).

In the current work we describe the thermal inactivation of BoNT/A, BoNT/A complex, BoNT/B and BoNT/B complex in cow's milk in a close-to-industry pasteurization process.

As an internal reference for the experimental set-up, we correlate the activity of the toxins with the enzymatic activity of ALP, thereby mimicking the industrial process where the loss of ALP activity is used to indicate correct pasteurization. Unexpectedly, our data show that under industrial pasteurization conditions the BoNT and BoNT complexes we analyzed are inactivated to 99.5% or more.

Material and methods

Milk. A batch of fresh, full-fat, raw bovine milk (pH 6.6) was purchased from a local retail store, divided into 10 mL-aliquots and stored frozen at -20°C.

Toxins and antibodies. The study was performed either using the purified 150 kDa BoNT/A1 (Hall A), BoNT/B1 (Okra B) or the corresponding BoNT complexes which were all purchased from Metabiologics Inc. (Madison, WI, USA). Monoclonal mouse antibody A1688 (IgG1, κ) was used to capture BoNT/A and BoNT/A complex, and monoclonal mouse antibody B279 (IgG2a, κ) to capture BoNT/B and BoNT/B complex in the sandwich ELISA (30). Antibodies were purified from hybridoma supernatants using HiTrap Protein G sepharose columns according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein concentration via absorbance at 280 nm using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). For subsequent studies, IgG antibodies were stored in phosphate-buffered saline (pH 7.3) at 1 mg mL^{-1}. For the detection of BoNT and BoNT complexes biotinylated polyclonal equine anti-BoNT (anti-A, B, E; Novartis Behring, Marburg, Germany) was used. The antibody was coupled to biotin according to manufacturer's instructions (EZ-Link Sulfo-NHS-LC-Biotin, Pierce, IL, USA). Biotinylated antibodies were stored in phosphate-buffered saline with 0.2% (w/v) BSA and 0.05% (w/v) NaN3.

Laboratory-scale pasteurization and measurement of ALP activity. Raw milk was thawed and kept at 4°C before and after thermal treatment. To mimic industrial milk pasteurization the raw milk was dispensed at volumes of 50 µL into thin-walled 0.2 mL PCR-vials (VWR, West Chester, PA, USA) and subjected to heat treatment in a MJ MiniOpticon Cycler (Biorad, Hercules, CA, USA) with a lid temperature of 99.9°C. At the rate of 2.5 K s^{-1} all samples were heated to 15°C maintained for 30 seconds, then heated to 72°C with a slope of 0.5 K s^{-1} and held at 72°C for 1, 5, 10, 15, 30, 60, 120, or 180 seconds, respectively. Subsequently, the samples were cooled to 15°C with a slope of 2.5 K s^{-1} and then to 4°C. Similarly-heated samples were pooled for subsequent analysis. Since the accuracy of the heat treatment delivered is a crucial point of the experimental setup, the thermal cycler was independently validated using a multichannel temperature acquisition system (BioRad, Hercules, CA, USA; http://www.cyclertest. com/prod_art.asp?product= 13&category=1). The results of the validation are shown in Supplementary Fig. S1: exemplarily shown for 70°C, a temperature variation of 69.6°C +/- 0.2°C was detected.

ALP was used as an intrinsic time-temperature integrator for the heat treatment of milk. The ISO standard (21) requires that pasteurization protocols lead to the inactivation of ALP activity below the 350 mU L^{-1} threshold. For standardized ALP measurement (standard ALP assay) the Fluorophos® method (Advanced Instruments Inc., Norwood, MA, USA) was used. Following the standard protocol, Fluorophos® substrate (2'-[2-Benzothiazolyl]-6'-hydroxybenzothiazole-phosphate) in Diethanolamine (DEA) buffer solution at pH 10 was used to detect the dephosphorylating activity of alkaline phosphatase. Two mL of Fluorophos® were heated to 38°C in a glass cuvette. After adding 75 µL of the milk the sample was vortexed and allowed to reach 38°C in the Fluorophos® 156 reader. Measurement of fluorescence was performed at an excitation wavelength of 440 nm and an emission wavelength of 560 nm. Fluorescence was measured every 30 seconds over a total of 120 seconds. Calibration of the system and calculation of the enzymatic activity of ALP were performed as described elsewhere (21).

In the miniaturized ALP measurement (mini ALP assay), 10 µL of milk samples were pipetted in triplicate on the bottom of FluoroNunc F96 MicroWell plates (Nunc, Langenselbold, Germany) which were preheated to 38°C. 100 µL of Fluorophos® substrate,

also heated to 38°C, were added to each sample cavity and immediately mixed with the sample by gentle pipetting. The fluorescence was measured at 38°C with an InfiniTE M200 monochromator (Tecan, Crailsheim, Germany) at an excitation wavelength of 440 nm and an emission wavelength of 560 nm. Fluorescence was measured every 30 seconds over a total of 120 seconds. Enzymatic activity of ALP was calculated with the mean fluorescence min^{-1} of the triplicates, multiplied by the amount of Fluoroyellow® in calibration solution B and divided by the calibration ratio and the sample volume. Due to device-specific limitations, the InfiniTE M200 from Tecan was only able to show a maximum of 100,000 mU L^{-1} in the fluorescence measurement of the miniaturized Fluorophos® method.

Toxin spiking. For the measurement of BoNT inactivation, 1 mg mL^{-1} of each toxin was diluted in raw milk to give a final concentration of 500 ng mL^{-1}. One mouse lethal dose (MLD) was defined as lowest total amount of toxin that kills 100% of all mice in an experiment. According to this definition, one MLD was determined by mouse bioassay as 5 pg for purified BoNT/A and BoNT/B, and 15 pg for BoNT/A complex and BoNT/B complex, respectively. These experiments have been performed with 5 mice for each of the four toxin preparations and have been highly reproducible (data not shown). Accordingly, 500 ng mL^{-1} 179 contained 100,000 MLD mL^{-1} for purified BoNT/A and BoNT/B and 33,333 MLD mL^{-1} 180 for BoNT/A complex and BoNT/B complex.

For each of the different time points indicated in the text, a total volume of 1000 µL at 500 ng mL^{-1} has been divided into 50 µl aliquots, heated as described in a thermal cycler and pooled again to 1000 µL. From this volume, 1) 3 x 10 µl have been used for the ALP assay, 2) 2 x 50 µl have been used for the ELISA and 3) either 100 µL (in the case of the purified neurotoxins) or 300 µL (in the case of the neurotoxin complexes) have been used for the mouse bioassay, corresponding to a total amount of 10,000 MLD each.

Enzyme-linked immunosorbent assay (ELISA). MaxiSorp microtiter plates (96F Nunc, Langenselbold, Germany) were coated with monoclonal antibody A1688/2 (anti-BoNT/A) or B279/5 (anti-BoNT/B) at 10 and 8.7 µg mL^{-1}, respectively, in phosphate-buffered saline (pH 7.2) at 4°C overnight (30). After blocking for 60 minutes, heated and non-heated samples with BoNT as well as negative controls were applied in duplicate, and incubated at 25°C for 120 minutes. Sample cavities were washed and incubated for 60 minutes with biotinylated polyclonal equine anti-BoNT/A, /B, /E antiserum (Novartis Behring, Marburg, Germany) at 60 µg mL^{-1}, followed by incubation with streptavidine-coupled horseradish peroxidase (Dianova, Hamburg, Germany) for 30 minutes at a dilution of 1:2500. TMB (3, 3', 5, 5'-tetramethylbenzidine; Sigma-Aldrich, Seelze, Germany) was used as a substrate and the average absorbance was measured at 450 nm minus absorbance of impurities at 620 nm wavelength.

Biological activity of BoNT measured by mouse bioassay. Mouse bioassay was used to estimate the biological potency of non-heated and heat-treated raw milk spiked with BoNT. The assay was performed with female BALB/c-mice, weighing between 15 and 21 g (35). BALB/c mice were raised under specific pathogen-free conditions at the German Federal Institute for Risk Assessment (Berlin, Germany) and were between 6 and 8 weeks old. For the experiments, mice were maintained under barrier conditions at the Robert Koch-Institut (Berlin, Germany). All animal experiments were performed in accordance with the German Animal Protection Law and were approved by the regional authority for health and social affairs (LAGeSo Berlin). Considering that 100,000 MLD mL^{-1} of purified BoNT and 33,333 MLD mL^{-1} BoNT complexes were spiked into raw milk before thermal treatment, a total amount of 10,000 MLD was injected into mice intraperitoneally (corresponding to 100 µL in the case of the purified neurotoxins, adjusted to 300 µl with equally pasteurized milk, or 300 µL in the case of the neurotoxin complexes, respectively). Negative control mice received raw milk without BoNT and positive controls received unheated raw milk with BoNT. Samples and controls of each time point were tested in duplicate. For dilutions used to investigate the activity of BoNT complexes after heating, again equally pasteurized milk was used. For the critical dilutions - 1:10- and 1:50-diluted samples (holding time of 1 and 15 seconds) and 1:10-diluted samples (holding time of 180 seconds), respectively - five mice were used per group. Injected mice were observed for typical botulism symptoms for up to 96 hours. When a wasp-like narrowed waist and immobility due to severe paralysis were observed, the mice were sacrificed. The absence of botulism symptoms indicated that no toxic activity remained. If mice that received samples with purified BoNT or BoNT

complexes showed no symptoms, the remaining MLD was considered to be less than one. In all other cases dilution factors allowed for the approximation of the remaining MLD.

Results

Establishment of close-to-industry, laboratory-scale pasteurization process. To set up a laboratory-scale pasteurization process, raw milk was subjected to thermal treatment at 72°C for 15 seconds. The efficacy of the thermal treatment was monitored by analyzing the milk alkaline phosphatase (ALP) activity as an intrinsic parameter of successfully completed pasteurization. To monitor the pasteurization process, ALP activity was measured using a standardized ALP assay (standard ALP assay), which is also regularly used in the dairy industry. According to internationally accepted standards, the activity of ALP after HTST pasteurization at 72°C for 15 seconds must drop below 350 mU L^{-1} (21). In order to find the right laboratory parameters and dimensions which would mimic the industrial process, we tested two different experimental set-ups: a fixed volume of 50 µl raw milk was heated either in a thermo mixer or in a thermal cycler. Using the thermo mixer, samples were subjected to 72°C for different times and then rapidly cooled using liquid nitrogen. Using the thermal cycler, samples were subjected to a heating curve up to 72°C, held at that temperature for different times and then brought down to 15°C using the in-built cooling system. As shown in Fig. 1, ALP activity from samples in the thermo mixer reached values below 350 mU L^{-1} after only several minutes at 72°C, whereas the thermal cycler showed values below the threshold already after a 15-second holding time at 72°C (Fig. 1 and Fig. 2A). Accordingly, monitoring the activity of ALP in the heated milk, we observed enhanced heat transfer using the thermal cycler. Also, it allowed for better process control with respect to the heating profile, thereby allowing us to compare the efficacy of the heating process and the industrial HTST pasteurization process directly. Consequently, the thermal cycler was used to heat the milk samples in all further experiments.

To measure ALP activity in milk spiked with BoNT in our small-scale laboratory set-up, the industrial standard ALP assay was downscaled from 2000 µL to a miniaturized ALP assay format (mini ALP assay), using 100 µL volume (see Material and Methods). Both assays were compared using unspiked milk heated with the thermal cycler (Fig. 2A). The standard ALP assay showed ALP activities typically found in raw milk, ranging from 200,000 to 500,000 mU L^{-1} ((33) and C. Egger, personal communication), slightly more than those found with the miniaturized method. After a 15-second holding time at 72°C, the ALP activity in milk was reduced to values below the 350 mU L^{-1} threshold. As shown in Fig. 2A, both methods led to an almost identical reduction of ALP activity below the indicated threshold at the same holding times. The miniaturized ALP assay, therefore, was used to monitor the loss of ALP activity during thermal treatment in all further experiments.

To exclude the possibility that milk pasteurization is altered by the presence of BoNT, raw milk and raw milk spiked with 100,000 mouse lethal dose (MLD) per mL of purified BoNT/A or BoNT/B, or 33,333 MLD mL^{-1} of BoNT/A complex or BoNT/B complex, respectively, were heated in parallel in a thermal cycler and measured with the mini ALP assay. The results presented in Fig. 2B show that ALP activities in heated milk were consistent, regardless of whether the toxin was present in the milk or not.

Based on the measurement of the intrinsic milk parameter ALP by both the standardized and the miniaturized ALP assay, the results show that heating for 15 seconds at 72°C in the thermal cycler is sufficient to obtain pasteurized milk. Thus, according to internationally accepted standards, our laboratory-scale method can be considered a close-to-industry pasteurization process that allows for direct comparison of heat transfer and protein activity.

Thermal inactivation of BoNT/A, BoNT/B, and BoNT complexes as determined by ELISA. To monitor the thermal inactivation of purified BoNT/A, BoNT/B and the corresponding complexes in milk, we spiked defined amounts of the toxins into raw milk. From the same samples we subsequently determined 1) the ALP activity using the mini ALP assay as indicated above, 2) the structural integrity of the toxins by sandwich ELISA specific for BoNT/A and for BoNT/B, and 3) the functional activity of the toxins using the mouse bioassay.

The performance of the two sandwich ELISA used in this study is indicated in Supplementary Fig. S2 and Table 1. Both ELISA are based on the combination of a monoclonal capture antibody (clone 1688/2 for BoNT/A; clone B279/5 for BoNT/B, (30)) with an equine anti-BoNT/A, /B, /E antiserum resulting in detection limits between 47 and 136 pg mL^{-1} for BoNT/A and BoNT/B or 558 and 698 pg mL^{-1} for the corresponding complexes, both in buffer and in raw milk (Suppl. Fig. S2, A and B, and Tab. 1). As shown in Fig. S2, both ELISA are able to discriminate between active and inactive BoNT (where "inactive BoNT" is equivalent to toxin that has been heated for 15 seconds at 72°C) over a range of about three orders of magnitude of concentration (Suppl. Fig. S2, C-F), therefore allowing us to reduce the number of animals used in this study.

After spiking the milk with 500 ng mL^{-1} purified BoNT/A, BoNT/B or the corresponding complexes (equivalent to 100,000 MLD mL^{-1} for the purified neurotoxins or 33,333 MLD mL^{-1} for the toxin complexes, respectively), we performed the laboratory-scale pasteurization as indicated above and measured the presence of the toxins after appropriate dilution of the milk samples. As shown in Fig. 3A and 3B, BoNT/A and BoNT/B as well as their respective complexes could be clearly detected in the spiked, unheated raw milk samples and were not present in the unspiked samples. Applying our heating protocol for different time points (1, 5, 15 and up to 180 seconds at 72°C) resulted in a dramatic reduction in the toxin-specific signal detected via sandwich ELISA, both for the purified neurotoxins and the neurotoxin complexes. In the case of the purified neurotoxins, a holding time of only 1 second at 72°C led to a complete loss of the ELISA signal (Fig. 3A and B). For the neurotoxin complexes, the ELISA signal sharply fell even after only a holding time of 1 second at 72°C. However, it was still detectable with approximately 20% signal intensity when compared to the spiked, unheated milk (Fig. 3A and B). For both BoNT complexes, a holding time of 180 seconds at 72°C was needed before the signal decreased to 1-4% of the original value. Even though the ELISA suggested a dramatic loss of BoNT integrity within the first few seconds of heating, a residual functional activity could not be excluded.

Thermal inactivation of BoNT/A, BoNT/B, and BoNT complexes as determined by mouse bioassay. We performed the mouse bioassay to quantify the residual toxic activity of purified BoNT and BoNT complexes spiked into milk after laboratory-scale pasteurization. Considering that initially 100,000 MLD mL^{-1} of purified BoNT and 33,333 MLD mL^{-1} BoNT complexes (serotype A and B each, see above) were spiked into raw milk before thermal treatment, a total amount of 10,000 MLD was injected into mice intraperitoneally. The application of unheated raw milk containing 10,000 MLD BoNT/A, BoNT/A complex, BoNT/B or BoNT/B complex to BALB/c mice led to typical symptoms of botulism, such as a wasp-like narrowed waist and subsequent paralysis.

Interestingly, all mice injected with milk spiked with purified BoNT/A or BoNT/B held at 72°C for 1 second or longer survived without showing any symptoms of botulism. Therefore, this experiment showed that the 10,000 MLD of BoNT originally spiked into milk were reduced to less than 1 MLD, indicating that the heat treatment reduced the toxic activity of purified BoNT/A and BoNT/B by more than 99.99% (Tab. 2).

Yet, when we applied thermally treated milk spiked with BoNT/A complex or BoNT/B complex instead of with the purified neurotoxins, all mice suffered from typical symptoms of botulism. Stepwise dilutions of the heated milk originally containing 10.000 MLD were used to determine the residual toxicity. A 1:50-dilution, corresponding to 200 MLD of the originally spiked milk held at 72°C for 15 seconds, was no longer toxic when injected into mice, demonstrating that less than 1 MLD was left from the injected 200 MLD. Therefore, the heat treatment reduced the toxicity by at least 99.5%. A similar calculation leads us to conclude that heat treatment at 72°C for 180 seconds reduces the toxicity of both BoNT complexes by more than 99.9% (Tab. 2).

Taken together, heating under standard milk pasteurization conditions - 72°C for 15 seconds - was sufficient to reduce the toxic activity of purified BoNT/A and BoNT/B by more than 99.99% and that of the corresponding BoNT complexes by more than 99.5%.

Discussion

The results presented in this paper show that current conditions of industrial HTST pasteurization are effective in reducing the toxic activities of both BoNT/A and BoNT/B by more than 99.99% and of the corresponding BoNT complexes by 99.5%. Consequently, these results could provide a significant contribution to scenarios modeling BoNT as a potential biowarfare agent.

Milk and milk-derived products are amongst the most consumed food products worldwide. Per capita milk consumption in 2007 was approximately 80-90 liters in the United States and Western European countries (19). For more than 100 years the thermal treatment of milk has been used effectively to inactivate pathogens that may be present in milk (10). Common heating processes are high temperature short time (HTST; 72°C, 15-16 seconds), extended shelf life (ESL; 80°C and 130°C, 1-5 seconds), and ultra high temperature (UHT; 135-150°C, 1-10 seconds).

Heat-treated milk has proven to be a safe food and no cases of "natural" foodborne botulism have been described from industrially processed milk. Nevertheless, even when milk production on farms meets modern standards on food quality and hygiene, the production process cannot be completely secured, making the supply chain vulnerable to bioterror attacks. One scenario that could be considered is the deliberate release of the agent in raw milk prior to pasteurization on the farm or while in transit to the dairy company.

For the current study it was important to consider the respective food matrix used for the experiments, in this case raw milk, since it has a major impact on the heat inactivation rate of BoNT (37). Early work by Scott and Stewart (1950) demonstrated that vegetable juice increased the heat stability of BoNT/A and BoNT/B, being protected by bivalent cations and organic acid anions present in the juice (36). Later, Bradshaw, Peeler & Twedt (1979) showed that BoNT/A and BoNT/B were more heat-stable in beef and mushroom patties than in a phosphate buffer at the same pH (5). Woodburn (1979) also observed increased heat stability of BoNT/A when 1% gelatin was added to a phosphate buffer 358 (42). Recently, it has been shown that the molten globule-like character of BoNT and the interaction with NAPs is responsible for varying physical stabilities at different pH values (6, 8). The data showed a stabilizing effect of NAPs on the purified neurotoxins. It is worth considering, that if the NAPs were damaged this could influence the stability of the whole complex and the oral toxicity. Based on the available data, it seemed conceivable that the actual stability of BoNT in milk during the pasteurization process cannot be directly extrapolated from other food matrices which were analyzed earlier. Rather measuring the actual stability of the toxins in milk appears critical for the generation of reliable numbers.

When comparing different experimental set-ups for the thermal treatment of milk, our experiments highlighted a second critical parameter, namely the importance of finding experimental conditions for heat transfer that mimicked the industrial process as closely as possible. Our data from the comparison of heating milk with a thermo mixer and with a thermal cycler suggested that strict compliance with pasteurization parameters is necessary to guarantee the degree of inactivation described above. While a thermo mixer involves heating with an isothermal heat source for a defined time, the thermal cycler allows for temperature-controlled heating with defined holding times, an approach which complies with the industrial HTST pasteurization process. Compared to ESL and UHT processing, HTST pasteurization applies less thermal load, and is also the most commonly used and mildest thermal treatment of milk, hence our decision to apply this process to our experiments. Unlike the industrial process which uses forced convective heat transfer, heating in a thermal cycler is achieved by free heat transfer. Yet, ALP, used as an intrinsic time-temperature integrator for the heat treatment of milk, allowed us to show that laboratory-scale pasteurization in a thermal cycler met dairy industry requirements for successful HTST pasteurization ((12), i.e. inactivation of ALP within 15 seconds at 72°C below a threshold of 350 mU/L). These results are also in accordance with findings of an earlier study that applied the same methodology of thermal treatment and measuring ALP activity when investigating the effect of pasteurization on herpes virus infectivity in milk (4). Retrospectively, it is not clear, if the classical work cited above (5, 36, 42) is as well-defined as our current work with respect to the heat transfer applied, the proven assignability to an

industrial process and the toxin preparations used (neurotoxin or toxin complexes; purity of the material).

Due to their high bioavailability BoNT complexes present the most toxic form of BoNT if ingested (9, 25), and the deliberate release of this form would be a worst-case scenario. For this reason, we spiked purified BoNT or BoNT complexes into raw milk prior to pasteurization. Subsequently, loss of structural integrity and toxic activity were determined by sandwich ELISA and mouse bioassay, respectively. Both the immunological and functional detection of BoNTs generated similar results, indicating a dramatic loss of protein structure and function. In contrast to the 68.4% heat inactivation of BoNT estimated by Wein and Liu (41), we were able to show in the mouse bioassay that even after only 1-second holding time at pasteurization temperature, the toxic activity of purified BoNT/A and BoNT/B fell by more than 99.99%. Similarly, we were able to show that BoNT/A complex and BoNT/B complex were inactivated by more than 99.5% under common pasteurization conditions. As observed before, BoNT complexes showed a slightly higher degree of stability; this was probably due to the stabilizing effect of associated NAPs (6, 8). In the case of the complex proteins, the sandwich ELISA did not completely reflect the functional assay; this might indicate that distinct epitopes of the BoNT complexes detected by ELISA were protected somewhat against thermal inactivation either by the accompanying complex proteins and/or by the interaction of the BoNT complex with milk components. However, considering the results of the mouse bioassay, the detected epitopes seem not to be directly linked to the toxic activity of the toxin. Although ELISA provides no information on the remaining toxic activity in the heated samples, it reflects the overall stability of the toxin structure under distinct heating conditions.

In the bioterror scenario described by Wein and Liu, 1 g of BoNT released into approximately 230,000 liters (50,000 gallons) of raw milk would lead to 3.2×10^4 possible victims, based on 68.4% inactivation of the toxin by heat treatment. However, our experimental results based on a close-to-industry pasteurization process showed 99.5% inactivation of BoNT complexes and 99.99% of purified BoNTs. Assuming a scenario in which 1 g of BoNT complex is deliberately released into raw milk and at least 99.5% of the toxin is inactivated by HTST pasteurization, the amount of biologically active toxin would be reduced to 5 mg in total. Applying our experimental data and assuming a linear relationship this would mean that following dilution in a 230,000-liter milk bulk tank, an average daily serving of 0.5 liters would contain approximately 11 ng of toxin at most. Considering the estimates for the human lethal oral dose of BoNT, this amount dramatically lowers the threat level of the widely discussed "BoNT in milk" scenario.

Our experimental data show that current conditions of HTST pasteurization are effective in reducing the toxic activities of BoNT/A, BoNT/B, and the corresponding BoNT complexes by more than 99.99% and 99.5%, respectively. Therefore, the HTST pasteurization process in the dairy industry dramatically reduces the risk of consumer harm even if larger amounts of BoNT were deliberately released into the milk supply chain. However, other complex food matrices or slight changes to the heating parameters might have a different effect on the stability of BoNT. As a result, we are unable to draw any general conclusions for other matrices and heating parameters. These will have to be the subject of further experiments.

Acknowledgments

References

1. **AOAC.** 2000. 991.24: Alkaline Phosphatase Activity in Fluid Dairy Products. Fluorometric Method.
2. **Arnon, S. S., R. Schechter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow, and K. Tonat.** 2001. Botulinum toxin as a biological weapon: medical and public health management. JAMA **285:**1059-70.
3. **Binz, T., and A. Rummel.** 2009. Cell entry strategy of clostridial neurotoxins. J. Neurochem. **109:**1584-1595.
4. **Bona, C., B. Dewals, L. Wiggers, K. Coudijzer, A. Vanderplasschen, and L. Gillet.** 2005. Short communication: Pasteurization of milk abolishes bovine herpesvirus 4 infectivity. J. Dairy Sci. **88:**3079-83.
5. **Bradshaw, J., J. Peeler, and R. Twedt.** 1979. Thermal inactivation of *Clostridium botulinum* toxins types A and B in buffer, and beef and mushroom patties. J. Food. Sci. **44:**1653-1657.
6. **Brandau, D., S. Joshi, A. Smalter, S. Kim, B. Steadman, and C. Middaugh.** 2007. Stability of the *Clostridium botulinum* type A neurotoxin complex: an empirical phase diagram based approach. Mol. Pharm. **4:**571-582.
7. **CDC.** 2005. 42 CFR Part 1003: Possession, Use, and Transfer of Select Agents and Toxins; Final Rule, 42 CFR Parts 72 and 73. Office of Inspector General.
8. **Chen, X., and Y. Deng.** 2007. Long-time molecular dynamics simulations of botulinum biotoxin type-A at different pH values and temperatures. J. Mol. Model **13:**559–572.
9. **Cheng, L. W., B. Onisko, E. A. Johnson, J. R. Reader, S. M. Griffey, A. E. Larson, W. H. Tepp, L. H. Stanker, D. L. Brandon, and J. M. Carter.** 2008. Effects of purification on the bioavailability of botulinum neurotoxin type A. Toxicology **249:**123-9.
10. **Czaplicki, A.** 2007. "Pure milk is better than purified milk". Soc. Sci. Hist. **31:**411-433.
11. **DasGupta, B. R., and D. A. Boroff.** 1968. Separation of toxin and hemagglutinin from crystalline toxin of *Clostridium botulinum* type A by anion exchange chromatography and determination of their dimensions by gel filtration. J. Biol. Chem. **243:**1065-72.
12. **EC.** 2005. Commission Regulation No 2074/200: laying down implementing measures for certain products under 853/2004 and 854/2004. Off. J. European Union **338:**27-59.
13. **FDA, and LQAB/NCIMS.** 2000. Form 2400k: Phosphatase test - Fluorophos ALP test system.
14. **Fischer, A., Y. Nakai, L. M. Eubanks, C. M. Clancy, W. H. Tepp, S. Pellett, T. J. Dickerson, E. A. Johnson, K. D. Janda, and M. Montal.** 2009. Bimodal modulation of the botulinum neurotoxin protein-conducting channel. Proc. Natl. Acad. Sci. U S A **106:**1330-5.
15. **Hatheway, C. L.** 1993. *Clostridium botulinum* and other Clostridia that produce botulinum neurotoxin., p. 3-21. *In* A. H. W. Hauschild and K. L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker Inc., New York.
16. **Hauschild, A. H. W.** 1993. Epidemiology of human foodborn botulism, p. 69-104. *In* A. H. W. Hauschild and K. L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker Inc., New York.
17. **Herrero, B. A., A. E. Ecklung, C. S. Streett, D. F. Ford, and J. K. King.** 1967. Experimental botulism in monkeys--a clinical pathological study. Exp. Mol. Pathol. **6:**84-95.
18. **Hill, K. K., T. J. Smith, C. H. Helma, L. O. Ticknor, B. T. Foley, R. T. Svensson, J. L. Brown, E. A. Johnson, L. A. Smith, R. T. Okinaka, P. J. Jackson, and J. D. Marks.** 2007. Genetic diversity among Botulinum Neurotoxin-producing clostridial strains. J. Bacteriol. **189:**818-32.
19. **IDF.** 2007. World Dairy Situation 2007. Bulletin IDF **432/2008**.
20. **Inoue, K., Y. Fujinaga, T. Watanabe, T. Ohyama, K. Takeshi, K. Moriishi, H. Nakajima, and K. Oguma.** 1996. Molecular composition of *Clostridium botulinum*

identification through toxin proteomics. Anal Chem **77:**6140-6146.

25. **Licciardello, J. J., C. A. Ribich, J. T. Nickerson, and S. A. Goldblith.** 1967. Kinetics of the thermal inactivation of type E *Clostridium botulinum* toxin. Appl. Microbiol. **15:**344-9.

26. **Lindstrom, M., K. Kiviniemi, and H. Korkeala.** 2006. Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing. Int. J. Food. Microbiol. **108:**92-104.

27. **Lund, B. M.** 1990. Foodborn illness: Foodborn disease due to *Bacillus* and *Clostridium species*. Lancet **336:**982-986.

28. **Marshall, K. M., M. Bradshaw, S. Pellett, and E. A. Johnson.** 2007. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. Biochem. Biophys. Res. Commun. **361:**49-54.

29. **Matsumura, T., Y. Jin, Y. Kabumoto, Y. Takegahara, K. Oguma, W. I. Lencer, and Y. Fujinaga.** 2008. The HA proteins of botulinum toxin disrupt intestinal epithelial intercellular junctions to increase toxin absorption. Cell. Microbiol. **10:**355-64.

30. **Pauly, D., S. Kirchner, B. Stoermann, T. Schreiber, S. Kaulfuss, R. Schade, R. Zbinden, M.-A. Avondet, M. B. Dorner, and B. G. Dorner.** 2009. Simultaneous quantification of five bacterial and plant toxins from complex matrices using a multiplexed fluorescent magnetic suspension assay. Analyst **134:**2028-2039.

31. **Peck, M. W.** 2006. *Clostridium botulinum* and the safety of minimally heated, chilled foods: an emerging issue? J. Appl. Microbiol. **101:**556-70.

32. **Popoff, M. R., and J.-C. Marvaud.** 1999. Structural and genomic features of clostridial neurotoxins., p. 174-201. *In* J. E. Alouf and J. H. Freer (ed.), The comprehensive sourcebook of bacterial protein toxins, 2. ed. Academic Press, London.

33. **Rocco, R. M.** 1990. Fluorometric determination of alkaline phosphatase in fluid dairy products: collaborative study. J. Assoc. Off. Anal. Chem. **73:**842-849.

34. **Rummel, A., T. Eichner, T. Weil, T. Karnath, A. Gutcaits, S. Mahrhold, K. Sandhoff, R. L. Proia, K. R. Acharya, H. Bigalke, and T. Binz.** 2007. Identification of the protein receptor binding site of botulinum neurotoxins B and G proves the double-receptor concept. Proc. Natl. Acad. Sci. U S A **104:**359-64.

35. **Schantz, E. J., and D. A. Kautter.** 1978. Microbiological methods: Standardized assay for *Clostridium botulinum* toxins. J. Assoc. Off. Anal. Chem. **6:**96.

36. **Scott, W., and D. Stewart.** 1950. The thermal destruction of *Clostridium botulinum* toxin in canned vegetables. Aust. J. Appl. Sci. **1:**200-207.

37. **Siegel, L. S.** 1993. Destruction of Botulinum Toxins in Food and Water p. 323-341. *In* A. H. W. Hauschild and K. L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker Inc., New York.

38. **Simpson, L. L.** 2004. Identification of the major steps in botulinum toxin action. Annu Rev Pharmacol Toxicol **44:**167-93.

39. **Singh, B. R.** 2006. Botulinum neurotoxin structure, engineering, and novel cellular trafficking and targeting. Neurotox Res **9:**73-92.

40. **Smith, T. J., J. Lou, I. N. Geren, C. M. Forsyth, R. Tsai, S. L. LaPorte, W. H. Tepp, M. Bradshaw, E. A. Johnson, L. A. Smith, and J. D. Marks.** 2005. Sequence Variation within Botulinum Neurotoxin Serotypes Impacts Antibody Binding and Neutralization. Infect Immun **73:**5450-5457.

41. **Wein, L. M., and Y. Liu.** 2005. Analyzing a bioterror attack on the food supply: the case of botulinum toxin in milk. Proc Natl Acad Sci U S A **102:**9984-9.

42. **Woodburn, M. J., E. Somers, J. Rodriguez, and E. J. Schantz.** 1979. Heat inactivation rates of botulinum toxins A, B, E and F in some foods and buffers. J. Food Sci. **44:**1658-1661.

Tables and Figures

Figure 1. Comparison of two different experimental set-ups for laboratory-scale pasteurization. A small volume (50 µl) of raw milk was heated either in a conventional thermo mixer (filled circles) or in a thermal cycler (open circles). For both set-ups, the reduction of ALP activity during continuous holding at 72°C for up to 300 seconds was monitored using the standard ALP assay.
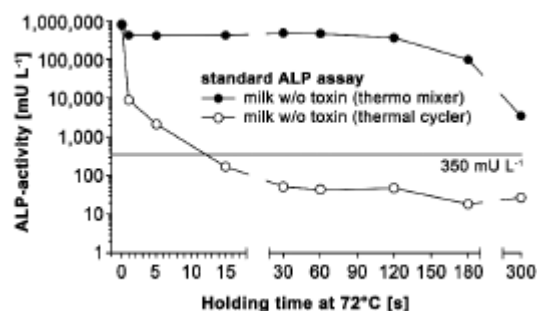

Figure 2. Reduction of ALP activity during heating of raw milk in a laboratory-scale pasteurization process using a thermal cycler. *(A)* ALP activity in raw milk and heated milk (each without toxin) was measured using the standard ALP assay (open circles) and compared to the activity measured with the mini ALP assay (open triangles). *(B)* ALP activity in unspiked raw and heated milk (open triangles) was measured with the mini ALP assay and compared to ALP activity in milk spiked with BoNT/A (blue triangles), BoNT/A complex (red triangles), BoNT/B (magenta triangles), and BoNT/B complex (green triangles). Data are shown for a holding time of 0 (unheated), 1, 15 and 180 seconds at 72°C. The threshold for successful pasteurization is 350 mU L^{-1}. Each line represents the mean of two independent experiments. Bars for standard deviation are shown above each symbol.
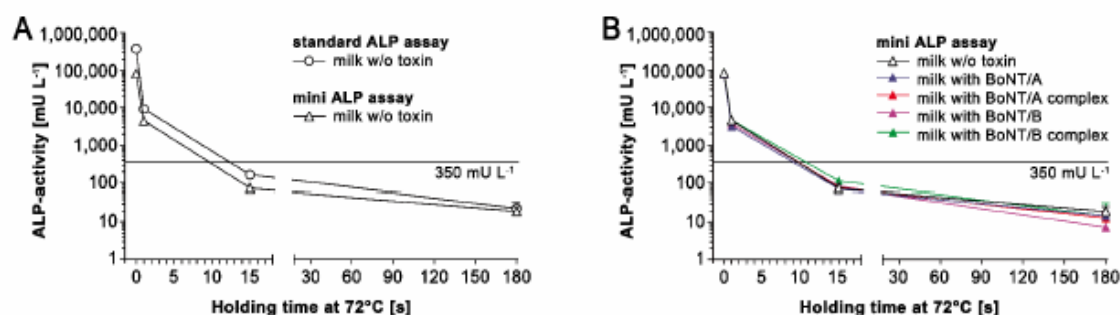
Figure 3. Structural integrity of BoNT/A, BoNT/B and corresponding BoNT complexes in milk during laboratory-scale pasteurization. Purified BoNT/A, BoNT/B or the corresponding complexes were spiked into raw milk as indicated in the text and subjected to our laboratory-scale pasteurization using a thermal cycler. After holding the samples for the indicated times at 72°C, sandwich ELISA were used to analyze them specifically for BoNT/A and BoNT/A complex *(A)* or for BoNT/B and BoNT/B complex *(B)*. Purified neurotoxins (BoNT) are depicted as open triangles, BoNT complexes as open squares. Unspiked milk samples (negative control) are shown as filled circles. Each line represents the mean of two independent experiments. Bars for the standard deviation are shown above each symbol.
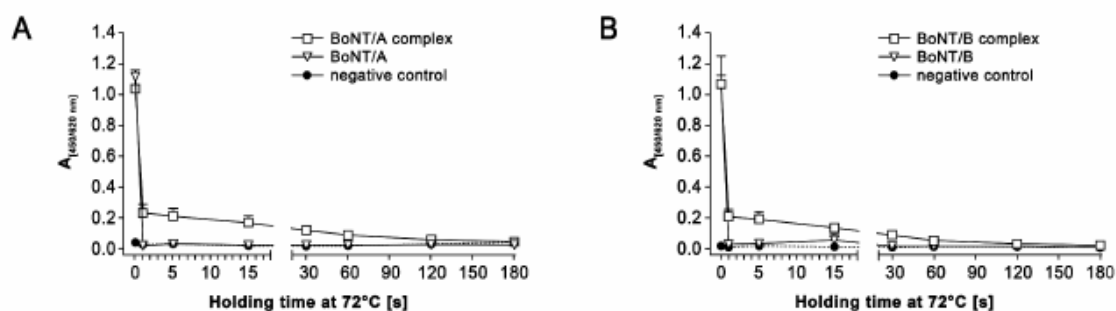
Table 1. Sensitivity of sandwich ELISA for the detection of BoNT/A, BoNT/B and the corresponding BoNT complexes in buffer and raw milk.

Toxin	LOD[a] in buffer[b]	LOD in raw milk
BoNT/A	53 ± 12 pg mL^{-1}	47 ± 9 pg mL^{-1}
BoNT/A complex	650 ± 221 pg mL^{-1}	558 ± 222 pg mL^{-1}
BoNT/B	102 ± 26 pg mL^{-1}	136 ± 51 pg mL^{-1}
BoNT/B complex	653 ± 184 pg mL^{-1}	698 ± 245 pg mL^{-1}

Limit of detection (LOD) was calculated on the basis of the arithmetic mean and the threefold standard deviation of 12-16 blank samples for three inter-assay standard curves. PBS containing 0.1% bovine serum albumin

Table 2. Toxic activity of BoNT/A, BoNT/B and the corresponding BoNT complexes in milk after pasteurization.

Heating at 72 °C	Dilution of BoNT-spiked milk	Mice alive / tested	MLD before / after heating	Reduction of toxic activity	Heating at 72 °C	Dilution of BoNT-spiked milk	Mice alive / tested	MLD before / after heating	Reduction of toxic activity
BoNT/A					**BoNT/B**				
unheated	-	0 / 2	10,000 / 10,000	-	unheated	-	0 / 2	10,000 / 10,000	-
1 s	-	2 / 2	10,000 / <1	>99.99%	1 s	-	2 / 2	10,000 / <1	>99.99%
5 s	-	2 / 2	10,000 / <1	>99.99%	5 s	-	2 / 2	10,000 / <1	>99.99%
15 s	-	2 / 2	10,000 / <1	>99.99%	15 s	-	2 / 2	10,000 / <1	>99.99%
30 s	-	2 / 2	10,000 / <1	>99.99%	30 s	-	2 / 2	10,000 / <1	>99.99%
BoNT/A complex					**BoNT/B complex**				
unheated	-	0 / 2	10,000 / 10,000	-	unheated	-	0 / 2	10,000 / 10,000	-
1 s	-	0 / 2			1 s	-	0 / 2		
	1:10	0 / 5				1:10	0 / 2		
	1:50	5 / 5	200 / <1	>99.5 %		1:50	0 / 5		
	1:100	2 / 2				1:100	5 / 5	100 / <1	>99.0 %
15 s	-	0 / 2			15 s	-	0 / 2		
	1:10	0 / 5				1:10	0 / 5		
	1:50	5 / 5	200 / <1	>99.5 %		1:50	5 / 5	200 / <1	>99.5 %
180 s	-	0 / 5			180 s	-	0 / 5		
	1:10	5 / 5	1,000 / <1	>99.9 %		1:10	5 / 5	1,000 / <1	>99.9 %