



A Monoclonal Enzyme-linked Immunoassay for the Detection of Botulinum Neurotoxin Type E

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Background and Aim: Botulinum neurotoxin Type E (BoNT/E), one of the most lethal toxin known, is the common contamination in fishery products or fish consumption that causes foodborne botulism. It is necessary to establish a sensitive and specific method for the detection of BoNT/E because of its extremely low lethal dose. **Methods:** In this study, a practical enzyme-linked immunosorbent assay for BoNT/E detection was developed. The assay is based on the sandwich format using monoclonal antibodies of two distinct specificities. An affinity-purified anti-BoNT/E light chain Mab (1E1) is utilized to adsorb BoNT/E from solution, and the second anti-BoNT/E heavy chain C terminus Mab (5E1) conjugated with peroxidase is then used to form sandwich complexes, and peroxidase allows color development and measurement of optical density at 450 nm. **Results:** Standard curves were linear over the range of 5–100 ng/ml BoNT/E. The limit of detection was below 10 ng/ml in phosphate-buffered saline buffer. The developed BoNT/E assay also showed no cross-reaction to Type A neurotoxin (BoNT/A) and Type B neurotoxin (BoNT/B). **Conclusion:** Herein, a sensitive and accurate ELISA for BoNT/E detection was presented. It has the potential to utilize in vivo BoNT/E analysis and contamination monitoring.

Key words: Botulinum neurotoxin type E, lethal toxin, foodborne botulism, enzyme-linked immunosorbent assay, monoclonal antibody

INTRODUCTION

Botulinum neurotoxins (BoNTs) are the most poisonous toxins in the world, and they sometimes cause the foodborne intoxication with high lethal rates. The seven different serotypes (A–G) of BoNT, each produced by distinct strains of *Clostridium botulinum* under anaerobic conditions,¹ cause flaccid muscle paralysis by blocking the release of a neurotransmitter, acetylcholine, at neuromuscular junctions.² Most of the human botulism is mainly caused by four types of A, B, E, and infrequently F.³ According to the annual summaries of the US CDC during 2010–2016, the average infection rate of different human foodborne botulism is accounted for 61% of Type A, 11% of Type B, 26% of Type E, 1% of Type F, and 2% of undefined cases.⁴ Among them, the

BoNT Type E (BoNT/E) is only commonly associated with the fishery products or fish consumption to induce foodborne botulism.^{5–7} Until now, it does become the public health emergency in many countries.^{5,6,8,9}

BoNTs have been extensively studied in both medical and basic research since its discovery in the 1880s. It consists of a ~50 kDa light chain and a ~100 kDa heavy chain linked through a disulfide bond. The heavy chain plays two roles of receptor binding and translocation across the membrane. The light chain acts as a zinc-dependent endoprotease to cleave polypeptides that are essential for exocytosis. Cleavage of these polypeptides leads to blockade of transmitter release and paralysis.^{10–12} The potential applications of BoNTs in therapy of

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human diseases characterized by hyperfunction of cholinergic terminals and use in many laboratories throughout the world as a useful tool in biomedical studies have revealed potential toxin contamination problems.¹³ The toxin is also considered a possible threat to selected populations as a potential agent of warfare or terrorist attack.¹⁴⁻¹⁶ An alternative bioassay using zebrafish has been developed in recent year, but the mouse bioassay is still the proved method for BoNTs detection.^{3,17,18} However, the procedures of mouse bioassay must take several days to complete. It is also recommended not to utilize animals for the diagnosis because of the ethical concerns. Therefore, the development of a sensitive detection method to monitor BoNTs would be always urgent.

Many sensitive polyclonal antibody-based BoNT/E immunoassay has been reported, but double monoclonal antibody (Mab)-based sandwich immunoassay has not been published.^{19,20} Mab has many desirable characteristics such as high affinity, permanent availability, and stability. The use of Mabs for preparation of enzyme conjugates, in place of polyclonal antibodies, also ensures labeling uniformity and permanent availability. Thus, we generated anti-BoNT/E Mabs and selected a pair of Mabs to develop a sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of BoNT/E.

MATERIALS AND METHODS

Materials

BoNT/E was purified from the culture of *C. botulinum* strains (ATCC 9564) by the methods of Gimenez and Sugiyama.²¹ Monoclonal antibodies (1E1 and 5E1) were produced in our laboratory and purified by protein G affinity column (Pharmacia, Uppsala, Sweden). Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Life Technologies GmbH (Eggenstein, Germany); cell culture plastics and ELISA plate were from Falcon Division, Becton Dickinson (Heidelberg, Germany) and Nunc GmbH (Wiesbaden, Germany). Collagenase, bovine serum albumin, HEPES, collagen, antibiotics, and cytostatics were purchased from SERVA (Heidelberg, Germany). All cell buffers were prepared with analytical grade chemicals from Merck (Darmstadt, Germany). o-Phenylenediamine (OPD) and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO, U. S. A.).

Hybridomas

The BoNT/E toxoid was produced by formaldehyde inactivation of whole BoNT/E. The toxin was incubated with 5% formaldehyde for 7 days at room temperature and dialyzed against phosphate-buffered saline (PBS) to remove

excess formaldehyde. Six BALB/c mice were vaccinated with toxoid (10 µg/mouse, intraperitoneal) and boosted 2 weeks later. After three times boost, these BoNT/E toxoid immunized mice were splenectomized. Their lymphocytes were fused to the mouse myeloma cell line FO using polyethylene glycol in a modification of the original method first described by Kohler and Milstein.²²

Western blot analysis and isotyping

Whole BoNT/E was run on a sodium dodecyl sulfate – 8% polyacrylamide gel under reducing conditions and transferred to nitrocellulose paper, using standard techniques. Supernatant from each hybridoma clone was tested by western blot for antibody to BoNT/E. Hybridomas secreted antibody can specifically recognize BoNT/E in a western blot. These hybridomas were doubly cloned, stabilized, and frozen. Supernatants and ascites were prepared from each cell line and purified by protein G for further characterization. The isotype of Mabs was determined using standard reagents that purchased from Sigma. An ELISA format with BoNT/E as the plate antigen (10 µg/ml; 100 µl/well) was employed. The supernatant was incubated on the plate and was followed by labeled anti-isotype antibody.

The development of monoclonal antibody-based enzyme-linked immunosorbent assay for botulinum neurotoxin Type E detection

The format of Mab-based sandwich ELISA for BoNT/E detection was configured as follows: polystyrene well-capture Mab-BoNT/E-detection Mab-HRP conjugate. According to the above format, the suitable capture Mab and detection Mab could be screened by different ELISA method.²³

Screening of capture monoclonal antibody

To select the best capture antibody, the ELISA format was performed as follow: an ELISA plate was precoated with anti-BoNT/E Mab (1 µg/ml). The wells were washed three times with the washing buffer (PBS containing 0.05% Tween 20) and blocked with 1% skim milk powder in PBS for 1 h. BoNT/E (1 µg/ml) was added to each well and incubated for 2 h at 37°C. The wells were washed three times with the washing buffer. After washing, polyclonal anti-BoNT/E antibody (1 µg/ml) was added and incubated for 1 h at 37°C. After washing the wells three times with the washing buffer, HRP-conjugated goat anti-rabbit IgG (1/3000 dilution) was added to each well and incubated at room temperature for 1 h. The plates were developed for 10 min at 25°C with OPD substrate in citrate buffer, pH 5.0, and the absorbance read at 450 nm on an ELISA reader.

Screening of detection monoclonal antibody

To select proper detection antibody, the ELISA format was changed as follow: an ELISA plate was precoated with BoNT/E (1 µg/ml) directly. The wells were washed and blocked. Anti-BoNT/E Mab (1 µg/ml) was added and incubated for 2 h at 37°C. After washing, HRP-conjugated goat anti-mouse IgG (1/3000 dilution) was added and incubated for 1 h at 37°C. After washing, the plates were developed for 10 min at 25°C with OPD substrate, and the absorbance read at 450 nm in an ELISA reader.

Conjugation of monoclonal antibody to horseradish peroxidase

Conjugation of the Mab to HRP was carried out by a modification of the method of Henning and Nielse.²⁴ Ten milligrams of peroxidase were dissolved in 2.5 ml of distilled water and mixed with 0.5 ml of 0.1 M sodium metaperiodate for 20 min at room temperature. The aldehyde was dialyzed against 0.001 M sodium acetate buffer, pH 4.4 at 4°C for 18 h. Fifty microliters of 0.2 M sodium carbonate buffer pH 9.5 was added to 10 mg HRP followed immediately by the addition of 2 mg of purified Mab. The mixture was stirred gently at room temperature for 2 h, and the reaction was stopped by the addition of 0.25 ml ascorbic acid (4 mg/ml). The conjugation was left standing for 24 h at 4°C to stabilize, then filter sterilized (0.22 µm) and stored at 4°C.

Assay of botulinum neurotoxin Type E by monoclonal antibody-based enzyme-linked immunosorbent assay

ELISA plate was precoated with monoclonal capture antibody (1 µg/ml). The wells were washed and blocked. Standard curves were prepared by diluting BoNT/E from 1 mg/ml stock solution with the appropriate volume of PBS. Diluted BoNT/E samples (100 µl/well) were added to each well and incubated for 2 h at 37°C. After washing, monoclonal anti-BoNT/E IgG-HRP conjugate (1:5000 dilution) was added and incubated for 1 h at 37°C. After washing, the plates were developed for 10 min at 25°C with OPD substrate, and the absorbance (A) read at 450 nm in an ELISA reader. Standard titration curves were constructed by plotting the absorbance value (mean of triplicate wells) against log₁₀ toxin concentration. Linear regression curves were constructed by plotting the absorbance value in 5–100 ng/ml (mean of triplicate wells) against log₁₀ toxin concentration.

Cross-reactivity of botulinum neurotoxin Type E monoclonal antibody-based enzyme-linked immunosorbent assay

Samples containing 1 µg/ml of BoNT/A and BoNT/B

were assayed by BoNT/E Mab-based ELISA for evaluating cross-reactivity.

RESULTS

Generation and characterization of anti-botulinum neurotoxin Type E monoclonal antibodies

Mice were immunized with BoNT/E toxoid. From one fusion, we obtained four high-affinity monoclonal anti-BoNT/E antibodies. The isotype of four Mabs was determined to be IgG1. The specificity of antibodies was characterized by electrophoresis and immunoblotting. Four Mabs, designed as 1E1, 1E3, 2E4, and 2E10, recognized BoNT/E light chain. Two Mabs, designed as 5E1 and 5E1, reacted with BoNT/E heavy chain [Table 1].

Screening of capture and detection antibodies

For assessment the hydrophobicity and antigen capture ability of Mabs, the Mabs were directly coated to the plate and tested its antigen capture ability. The results were shown in Figure 1. The Mab 1E1 showed the best antigen capture ability. For comparing the toxin-binding affinity of Mabs in sandwich ELISA, BoNT/E was coated to the plate and tested its antibody-binding affinity. Data were shown in Figure 2. The Mab 5E1 had the highest affinity to BoNT/E. Therefore, we selected Mab 1E1 as a capture antibody and Mab 5E1 as a detection antibody which were conjugated to HRP to develop Mab-based enzyme immunoassay system [Figure 3].

Standard curves

The toxin titration analysis showed a sigmoid titration curve over the range of 0–500 ng/ml BoNT/E [Figure 4]. The curve did not differ significantly when the assay was performed in the assay buffer, human urine diluted 1:10 in assay buffer, or human serum diluted 1:50 in assay buffer. In all of matrices, standard curves were linear in the range of 2.5–100 ng/ml BoNT/E ($r > 0.99$) [Figure 4, insert], and spiked samples as low as 10 ng/ml BoNT/E were accurately quantified.

Table 1: The properties of Mabs against BoNT/E

Monoclonal antibodies (Mabs)	Isotype	Chain recognized by Mab
1E1	IgG1	Light
1E3	IgG1	Light
2E4	IgG1	Light
2E10	IgG1	Light
5B1	IgG1	Heavy-C
5B3	IgG1	Heavy-C

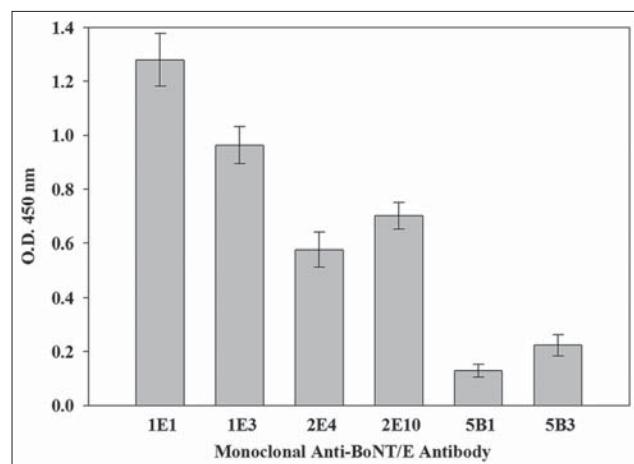


Figure 1: Capture monoclonal antibody screening. The enzyme-linked immunosorbent assay format was monoclonal antibody-botulinum neurotoxin Type E-rabbit anti-botulinum neurotoxin Type E IgG-goat anti-rabbit-horseradish peroxidase conjugate. The assay was performed as described in materials and methods. Bars are means of triplicate determinations. Control is irrelevant monoclonal antibody

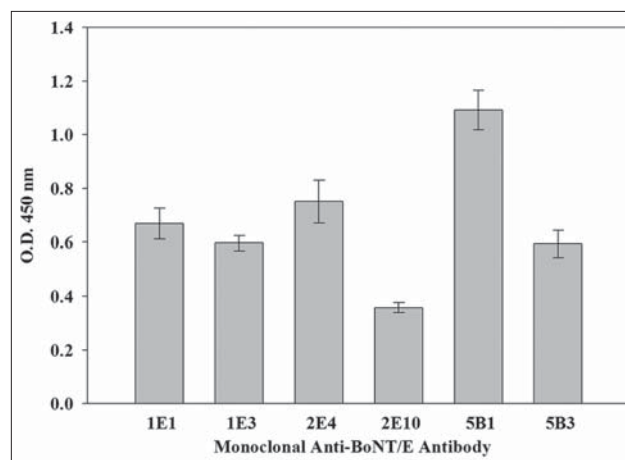


Figure 2: Detection monoclonal antibody screening. The enzyme-linked immunosorbent assay format was botulinum neurotoxin Type E-monoclonal antibody-goat anti-mouse-horseradish peroxidase conjugate. The assay was performed as described in material and methods. Bars are means of triplicate determinations. Control is irrelevant monoclonal antibody

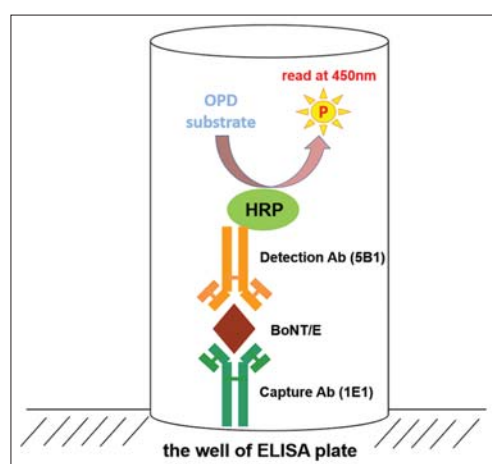


Figure 3: Scheme of monoclonal antibody-based sandwich enzyme-linked immunosorbent assay for botulinum neurotoxin Type E detection. A dual monoclonal antibody-based enzyme immunoassay was developed in this study. The monoclonal antibodies 1E1 and 5B1 were selected as the capture and detection antibody individually. The chemiluminescence signals can be read at 450 nm when o-phenylenediamine substrate was catalyzed with horseradish peroxidase (labeled on monoclonal antibody 5B1) after botulinum neurotoxin Type E detection

Cross-reactivity of botulinum neurotoxin Type E monoclonal antibody-based enzyme-linked immunosorbent assay

BoNT/A, BoNT/B, and BoNT/E are most frequently associated with human botulinum.² These BoNTs exhibit 30%–60% sequence identity.³ Therefore, the cross-reactivity of BoNT/E Mab-based ELISA with BoNT/A and BoNT/B was also examined. When 0–500 ng/ml of BoNT/A or BoNT/B

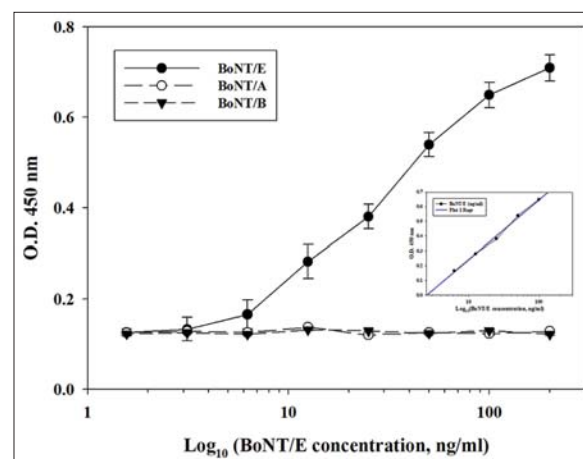


Figure 4: Standard enzyme-linked immunosorbent assay titration curves for botulinum neurotoxin Type E detection. The assay was performed as described in materials and methods, in phosphate-buffered saline (●) botulinum neurotoxin Type A (○) and botulinum neurotoxin Type E (▼). Points are means of triplicate determinations

was tested, linear (approach to background) titration curves were revealed in Figure 4. These results of developed assay showed no cross-reaction to BoNT/A and BoNT/B.

DISCUSSION

Hybridoma producing monoclonal antibodies against BoNT/E were generated by fusing mouse myeloma cell line FO with spleen cell of BALB/c mice immunized with BoNT/E toxoid. Six Mabs were selected from hundred others for their high antibody titer. To develop a monoclonal-based BoNT/E immunoassay system, these monoclonal antibodies were

screened for suitability as a capture or detection antibody by ELISA methods. Capture antibody must have strong hydrophobicity and good antigen-binding affinity, so that can be absorbed well by polystyrene ELISA plate, a very hydrophobic material, and maintain high antigen capture ability. Detection antibody must own high stability and antigen-binding affinity, so that can resist severe enzyme conjugation reaction and present good antigen detection ability. A monoclonal anti-BoNT/E light chain, identified as 1E1, had the strongest hydrophobicity, which was coated to the ELISA plate as a capture antibody. Another monoclonal anti-BoNT/E heavy chain, named 5E1, had the highest affinity, which was conjugation to HRP as a detection antibody. These two antibodies recognized different epitopes located in separated toxin structure domain. Using this pair of antibodies, we can obtain the optimal detection sensitivity.

We present here a simple, accurate, colorimetric, and monoclonal-based capture ELISA for BoNT/E assay. The detection limit is 10 ng/ml, equivalent to the sensitivity of polyclonal antibody-based assay.¹⁷ The routine assay can be performed in about 3 h if one starts with precoated plates. Although the assay sensitivity lower than the reported amplified or chemiluminescence immunoassay,^{25,26} we consider that this assay is sufficiently sensitive to support *in vivo* BoNT/E distribution analysis and in field BoNT/E contamination monitoring.²⁶

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Nil.

Conflicts of interest

There are no conflicts of interest.

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