Original Article

Development of An ICR Mouse Bioassay for Toxicity Evaluation in Neurotoxic Poisoning Toxins-Contaminated Shellfish

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Abstract

Objective To develop an ICR (female) mouse bioassay (MBA) for toxicity confirmation and evaluation of neurotoxins (brevetoxins)-contaminated shellfish.

Methods Brevetoxins (BTX-B) as a causative agent of neurotoxic shellfish poisoning (NSP) under different shellfish matrices were intraperitoneally injected at different doses into mice to study their toxic effects and to differentiate the range of lethal and sublethal dosages. Their sensitivity and specificity were analyzed with 2 competitive ELISA kits for quantitative determination of standard BTX-B and dihydroBTX-B under different shellfish matrix-diluent combinations. Detection rates of MBA and two antibody-based assays for BTX-B from field NSP-positive shellfish samples were compared.

Results BTX-B could be detected in shellfish tissues at concentration of 50-400 μg/100 g under shellfish matrix-Tween-saline media, which were appropriate to identify toxic shellfish at or above the regulatory limit (80 μg/100 g shellfish tissues). The LD_{50} identified was 455 μg/kg for BTX-B under general shellfish matrices (excluding oyster matrices) dissolved in Tween-saline. The presence of shellfish matrices, of oyster matrices in particular, retarded the occurrence of death and toxicity presentation in mice. Two antibody-based assays, even in the presence of different shellfish matrix-diluent combinations, showed acceptable results in quantifying BTX-B and dihydroBTX-B well below the regulatory limit.

Conclusion The two ELISA analyses agree favorably (correlation coefficient, $r \geq 0.96$; Student's *t*-tests, $P > 0.05$) with the developed bioassay.

Key words: Antibody-based assay; Brevetoxin; ICR female mice; LD_{50}; Mouse bioassay; Neurotoxic shellfish poisoning

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INTRODUCTION

Neurotoxic shellfish poisoning (NSP) is a marine biotoxin-induced illness due to consumption of filter-feeding bivalve shellfish contaminated with heat-stable methylated cyclic polyether neurotoxins known as brevetoxins. In general, the toxins can be classified into two general groups, namely A- and B-type, based on their polyether backbone structures[1-6]. A-type (type-1) containing 10 fused cyclic ether rings includes PbTx-1, -7 and -10 while B-type (type-2) containing 11 fused rings includes PbTx-2, -3, -5, -6, -8, and -9. PbTx-1 is the most potent among all the toxins. The most common brevetoxins found in the field are PbTx-2 (BTX-B) and PbTx-3 (dihydroBTX-B). The

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former is the most abundant and the latter may be produced from metabolic reduction of BTX-B by some shellfish species[7-12]. Other brevetoxin metabolites such as BTX-B1–8 have also been isolated and identified in some shellfish species[8,13-14]. Their toxic effects on humans are elicited by the activation of site 5 of the alpha subunit of voltage-sensitive sodium channels in nerve cells causing uncontrolled influx of sodium ions and depolarization of nerve membrane[15-16]. Clinical features of NSP in humans are characterized by a cluster of gastrointestinal and neurological symptoms such as abdominal pain, ataxia, diarrhea, dizziness, myalgia, nausea, paresthesias, vomiting, and reversal of heat and cold sensation[17-19], which are similar to those clinical implications from ciguatera fish poisoning[20-21]. No case of human mortality from brevetoxicosis is available, but a number of serious illness requiring hospitalization have been reported[12,18-19]. NSP incidents can also have negative impacts on shellfish businesses[22-23]. Since the first identification of these neurotoxins in shellfish from the human poisoning outbreak in New Zealand in 1992-1993, the mouse bioassay recommended by the American Public Health Association (APHA) for NSP (BTX-B equivalent level) (referred to as APHA mouse bioassay) has been a key routine detection method used by monitoring and regulatory agencies for protecting against this type of food poisoning[24-28]. In addition to the use of this biological assay, structural-based assays (enzyme-linked immunosorbent assay, ELISA), functional-based assays (receptor binding assay, RBA) and chemical-based analysis (liquid chromatography mass spectrometry, LCMS) have been considered as potential alternatives for detection of brevetoxins with a greater sensitivity and efficiency in comparison with the traditional mouse bioassay[19, 29-32]. However, the major weakness of these advanced methods lies in the fact that they cannot directly assay the toxicity of shellfish contaminated with brevetoxin(s). Cross-reactivity with other non-toxic brevetoxin metabolites and/or non-brevetoxin chemicals in the immunoassay is possible[7]. Moreover, some key toxins such as BTX-B4 (the most potent BTX found in shellfish) or other fatty acid forms have not been well recognized by immunoassay[33]. Therefore, at present, APHA mouse bioassay is still the only validated gold standard assay and is relatively fail-safe for screening the combined brevetoxins (all parent and metabolite compounds) toxicity in shellfish extracts. In order to provide a more comprehensive and efficient determination of brevetoxins in shellfish for routine operation, a rapid immunoassay coupled with this standard bioassay for toxicity confirmation is desirable to provide a two-tiered analytic approach for NSP toxins in shellfish. This can help to reduce the amount of experimental animals used in the screening test, while still providing a reasonably practicable public health protection against NSP outbreaks. It should be noted, however, that there is no validated NSP rapid detection assay recommended for routine screening of shellfish samples so far. Furthermore, some difficulties have been encountered when adopting the official APHA mouse bioassay method for toxicity screening or confirmation in brevetoxins-contaminated shellfish. The major drawbacks include the requirement of particular strain of mice (Swiss-Webster) for the test, solubility problems of extracts with the traditional delivery vehicle (cottonseed oil) and frequent syringe clogging when mice are injected with extracts dissolved in that vehicle[27]. These shortcomings significantly hinder operational application of this NSP mouse bioassay in laboratory tests and related researches.

In the present study, an ICR mouse bioassay using 5% Tween 60/0.9% saline (Tween-saline) as the delivery vehicle instead of the conventional cottonseed oil was developed for evaluating the levels of brevetoxins (μg BTX-B equivalent/100 g of shellfish tissues) in contaminated shellfish. In order to validate the efficacy of the bioassay, two commercially available competitive antibody-based methods were adopted to compare the results with those determined by the bioassay. Basic quantitative tests with brevetoxin standards (BTX-B and dihydroBTX-B) were conducted on the kits to assess the percentages of toxin detection by the kits with known toxin concentrations applied. The aim of this study was to establish a NSP mouse bioassay using a local mouse strain (ICR) for toxicity confirmation and evaluation in brevetoxin-contaminated shellfish previously identified from laboratory screening test(s). Some shortcomings from APHA NSP mouse bioassay have been removed, and as a result the applicability of this assay in laboratory operation has been strengthened.

MATERIALS AND METHODS

Chemicals and Brevetoxin Standards

Pure brevetoxin-B (BTX-B or PbTx-2) and dihydrobrevetoxin-B (dihydroBTX-B or PbTx-3)

standards (Calbiochem and Latoxan, the purity ≥95%) were obtained from Sigma-Aldrich (St. Louis, MO), Latoxan (Valence, France) and Marbionc (Wilmington, NC) respectively. Chemicals used for MARBIONC® NSP immunoassay included reagent A (antigen) (RA), reagent C (anti-brevetoxin antibodies) (RC) and reagent D (secondary antibodies) (RD) which were purchased from MARBIONC (Wilmington, NC). Phosphate buffered saline (PBS, pH7.4), gelatin from porcine skin (type A), liquid substrate 3,3', 5,5'-Tetramethylbenzidine (TMB), PBS-Tween 20 (PBS-T) and sulfuric acid (18M, ACS) were purchased from Sigma-Aldrich (St. Louis, MO), and SuperBlock® dry blend blocking buffer in TBS (RB) was obtained from Thermo Scientific Pierce (Rockford, IL). Reagents were prepared for the MARBIONC® NSP immunoassay in accordance with the brevetoxin ELISA instructions provided by MARBIONC.

Shellfish Sample Collection and Preparation

Raw shellfish samples (Cl: clams; Co: conches; Mu: mussels; Oy: oysters; Sc: scallops) with a mass of at least 200 g tissues were collected from markets in Hong Kong, and kept frozen or at 4 °C. All frozen samples were thawed at 4 °C prior to solvent extraction. Shellfish samples identified as "not detectable" by ICR (female) mouse bioassay and the two competitive antibody-based kits in this study were used for BTX-B spiked tests in the bioassay to investigate toxicity response(s) of ICR mice to different doses of BTX-B dissolved in various non-toxic shellfish matrix-Tween-saline media, and BTX-B and dihydroBTX-B spiked tests were used with two ELISA kits to investigate their sensitivity and specificity (% of toxins detection) in detecting toxins (under non-toxic shellfish matrix-diluent and solely diluent mediums) at mice subsymptomatic levels. Eight brevetoxin-positive shellfish samples including 4 clams (Cl1(MN)-Cl4(MN)), 3 conches (Co1(MN)-Co3(MN)) and 1 conch visceral (Co1(MN)vis) samples were supplied by Mote Marine Laboratory (Sarasota, Florida) for NSP-positive control and comparative study on results between our ICR mouse bioassay and the two antibody-based detection kits.

For processing the shellfish samples, the outer shell was cleaned with tap water. Shellfish tissue (150-200 g) was carefully removed and rinsed in deionized water to be free of any shell debris, sand and other foreign materials. The rinsed tissues were put in a sieve to drain for 5 min, and then homogenized in an electric blender for another 5 min until homogeneous slurry formed. For canned shellfish samples containing tissues and liquid, both portions were combined in proportionate quantities before homogenization.

Extraction of NSP Toxins from Shellfish Samples for Mouse Bioassay

Shellfish homogenates (50/100 g portions) were extracted with anhydrous diethyl-ether according to the official method recommended by American Public Health Association (APHA)[24-26,28]. Portions (50 g) were recommended for extraction because it would be easily handled for routine testing procedures and the evaporation process for the final ether extract would be faster. The remaining homogenates were stored in a -20 °C freezer for further analysis. Sodium chloride (NaCl, 2.5 g) and concentrated hydrochloric acid (HCl, 0.5 mL) were added into the 50 g homogenates. The acidified mixture was then boiled and cooked for 5 min with frequent stirring. The cooked mixture was cooled in a room temperature/ice-bath before extraction with anhydrous diethyl-ether using the American Chemical Society (ACS) grade level. Duplicate portions of homogenate samples were further extracted by another ACS grade and a non-ACS grade anhydrous diethyl-ether to compare the extraction performance of both grades of solvents. Three diethyl-ether extraction solvents, namely 1 non-ACS grade (solvent A) and 2 ACS grades (solvents B and C) were tested. No significant difference was found in component specification between the solvents except that information on alcohol, carbonyl and color conditions were not specified for non-ACS ether, and its stabilizer (butylated hydroxytoluene (BHT), ~5 ppm, 0.0005%) was about 5-10 times higher than those of the two ACS ether (solvent B: 1 ppm; solvent C: 0.5 ppm). The 50 g cooked mixture was extracted (100% w/v) with 50 mL of anhydrous diethyl-ether in a Teflon centrifuge bottle for 3-5 min (with vigorous shaking) before centrifugation at 2000×g for 15 min. The upper clear diethyl-ether phase (brown/green/yellow in color) was collected into a 600-mL LABCONCO® flat-bottom tube. The remaining residue was extracted 3 times more with diethyl-ether until the total amount of solvent used was 200 mL. Four portions of the upper ether phases were pooled in the flat-bottom tube to evaporate using a RapidVap N2 evaporation system supplied with filtered air and continuous gentle shaking. The dried diethyl-ether extract was then reconstituted in a small volume of anhydrous diethyl-ether, transferred to a 20-mL scintillation vial carefully using a glass pipette for final air-drying under a

gentle warming (37 °C) supply until the weight of extract was constant. Five percent Tween 60/0.9% saline (Tween-saline) was added to the dried extract until the final weight was gravimetrically brought up to 5 g (the volume of shellfish matrix-Tween-saline mixture was 5 mL). The mixture (shellfish matrix-Tween-saline medium) was sonicated and well mixed for 3 min until a homogenous extract mixture formed. In order to compare the solubility and absorption of Tween-saline with traditional vehicle (cottonseed oil) used in the official NSP bioassay, duplicate shellfish sample extracts were individually dissolved in either delivery vehicle to study the most appropriate one for routine laboratory use.

Mouse Bioassay

ICR (CD-1) strain female mice weighing 19-21 g (20±1 g) were obtained from the Laboratory Animals Service Centre, The Chinese University of Hong Kong to perform NSP bioassay. The mice were housed and acclimated in a controlled-environment (~24 °C, 54%-56% humidity, a 12-h:12-h light-dark cycle) for 1 day before the experiment with free access to standard rodent feed (PMI Nutrition International Inc., USA) and tap water. The NSP ICR mouse bioassay study was carried out by a designated laboratory operator (X). NSP symptoms of mice were verified by another laboratory operator (Y) without knowing details on dosages applied and sample information of tests.

One milliliter of the prepared extract mixture (10 g shellfish tissue equivalents) was subsequently heated to 37 °C and intraperitoneally (i.p.) injected into female ICR mice for toxin analysis. Then, behavior and rectal body temperature of the mice were closely monitored (first at 30-min and then 1-h intervals) for 6 h. The experimental setup was recorded with a video camera for a total of 24-48 h in order to determine the mice survival time and toxic symptoms beyond 6 h of close monitoring period. NSP (BTX-B) symptoms (lethal and sublethal doses) of mice compared with normal mice behavior and non-NSP toxic symptoms are listed in Table 1. All experimental mice used in the study were sacrificed in strict compliance with the stipulated ethical standards and guidelines of Department of Health, Government of Hong Kong, China.

Calibration and Toxicity Analysis of BTX-B under Shellfish Matrix Media on ICR Mice

LD_{50} of local mice species (ICR female mice) was determined by analyzing the relationship between injected dose (*C*) (ordinate) and dose over survival time (*C*/*T*) (abscissa) using the derived linear equation $C = a + b\,(C/T)$, where *C* represents dosage in mg/kg, *T* is survival time in minutes, the constants

Table 1. Normal Behaviors, Common NSP (BTX-B), and Non-NSP Toxic Symptoms/Behaviors in Mice

Normal Mice[a] Behaviors:	NSP (BTX-B) Symptoms (Lethal Dose): NSP(LD)	NSP (BTX-B) Symptoms (Sublethal Dose): NSP(SLD)	Non-NSP[b] Toxic Symptoms: N-NSPTS
Active	Body convulsions	Slight tail convulsions	Diarrhea (optional)
	Tail convulsions	Slight labored breathing	Weakness of limbs
	Hind leg paralysis	Hind leg paralysis	Prolonged lethargy
	Labored breathing	Walking imbalance	Walking slowly
	Gasping (with/without chewing sound)	Prolonged lethargy (optional)	Occasional respiratory spasms (optional)
	Body spasms	Occasional body spasms	Uncomfortable[c]
	Walking imbalance		
	Falling over		
	Death		
	Eyes darken after death		

Note. Symptoms/behaviors were applicable to mice injected intraperitoneally with BTX-B dissolved in shellfish-matrix-Tween-saline or solely Tween-saline medium. NSP symptoms (lethal and sublethal doses) observed in mice injected with BTX-B were comparable to those injected with dihydroBTX-B (data not shown). [a]: mice injected intraperitoneally with solely Tween-saline (5% Tween 60/0.9% saline) or cottonseed oil showed normal behaviors as uninjected mice and mice moving about in a playful fashion. [b]: unknown non-NSP toxin(s)/substance(s). [c]: no distinct sign, but body stretch was observed occasionally.

a and *b* are the asymptotes of hyperbola which represent the extrapolated smallest dose that kills mice at an infinite time and the minimum survival time of mice, respectively[34-35]. An intercept on y-axis of the linear relationship between (*C*) and (*C*/*T*) was determined as an evaluated value of LD_{50}. This method, as compared with traditional LD_{50} determinations, allows the use of fewer experimental mice but still produces a reliable value of the LD_{50}[34].

The calibration equation log (MU)=a log (1+1/*T*)+b derived from ciguatoxin mouse bioassay calibration[36-38] was adopted to establish a linear calibration for BTX-B, where MU and *T* represent the number of mouse units and survival time (minutes), respectively, for each mouse, and a and b represent the slope and y-intercept of the curve, respectively. The quantity of BTX-B in different shellfish extracts (toxins spiked into different shellfish matrices dissolved in Tween-saline) was evaluated by determining the dose (log MU) versus time-to-death [log (1+1/*T*)] relationship from female ICR mice using our established calibration curve. The toxin in Tween-saline (5% Tween 60/0.9% saline) was also studied to compare the response and survival time of mice with those under different shellfish matrices. Dosages between 0.0025 and 0.04 mg of BTX-B per mL of delivery vehicle were intraperitoneally injected into mice to study their toxic effects and to differentiate the range of lethal and sublethal dosages. A linear regression of the mice lethal dose for BTX-B using (log MU) against [log (1+1/*T*)] was established as a calibration curve for ICR mice (female) in order to determine the toxicity of BTX-B equivalent values (μg of BTX-2 equivalent per 100 g of shellfish tissues) in field shellfish samples. For statistical analysis, an unpaired Student's *t*-test was used for comparing the toxicity expression (survival times) between mice being injected with different doses of individually BTX-B under shellfish matrix-Tween-saline media (shellfish matrices with vehicle Tween-saline) and vehicle only (Tween-saline). $P<0.05$ was considered statistically significant between two groups of samples.

Analysis of Antibody-based Detection Assays for BTX-B and DihydroBTX-B

Two competitive ELISAs were studied for their performance on the detection of brevetoxins (BTX-B and dihydroBTX-B) in shellfish matrix-diluent and solely diluent media. Both ELISA studies were performed separately by two independent laboratory operators (X and Y) while operator Y was blind to the results of the ELISA test performed by X before completion of this study.

In the Abraxis® ELISA kit, brevetoxins in a sample competed with brevetoxin-horse radish peroxidase conjugates (brevetoxin-HRP conjugate) for the binding sites of sheep anti-brevetoxin antibodies immobilized in the wells of a microtiter plate. After the wells were washed with buffer, a color signal was produced by addition of the substrate solution. Color intensity (blue) was inversely proportional to the concentration of brevetoxins present in sample. The color reaction was terminated after a specified time with a diluted acid and the color (yellow) produced was measured with a spectrophotometer (450 nm). The concentration of brevetoxins in the sample was measured by interpolation using the standard curve (Abraxis® PbTx-3/dihydroBTX-B standards) constructed with each run. In the Marbionc® ELISA kit, brevetoxins in a sample competed with brevetoxins immobilized in the well of microtiter plate for the binding sites of goat anti-brevetoxin antibody. A secondary antibody with brevetoxin-HRP conjugate was added followed by incubation and washing. A substrate solution was added for color generation (blue in color) which was terminated by adding acid after a specified time. The intensity of the color produced (yellow, 450 nm) was inversely proportional to the concentration of brevetoxins present in the sample. The concentration of the sample was measured by interpolation using the standard curve (commercially available PbTx-3/dihydroBTX-B standards) constructed with each run. Both ELISA kits were performed strictly in accordance with the procedures and recommendations suggested by their manufacturers. The expiration dates and information of the kits were checked and recorded in each test for traceability purposes.

Extraction of Shellfish Samples for Antibody-based NSP Detection Kits

One gram of the prepared homogenized shellfish sample was placed in a Wheaton 40-mL flat-bottom glass vial. Nine milliliters of 90% aqueous methanol was then added to perform extraction. The mixture was shaken vigorously in the capped glass vial for 2 min with occasional sonication before centrifugation at 3000×g for 10 min. The supernatant obtained was then analyzed by the two competitive antibody-based detection kits for NSP toxins.

Assay Procedures of Competitive Antibody-based Kits for NSP Detection

For the Abraxis® ELISA kit, 20 μL of shellfish sample extract was collected from the supernatant, and diluted at 1:50 with Abraxis® sample diluent to form diluted shellfish sample extracts (shellfish matrix-diluent). In order to study the matrix effect of shellfish extracts on the kit performance, brevetoxin standards (BTX-B or dihydroBTX-B) were individually spiked into the diluted supernatant for quantitative tests. For operating the Abraxis® kit, 50 μL of the Abraxis® standard solution and the diluted supernatant (i.e. diluted shellfish sample extracts) were added in duplicate into a flat-bottom 96-well Abraxis® microtiter plate coated with sheep anti-brevetoxin antibodies. Fifty microliters of Abraxis® brevetoxin-HRP conjugate solution was added to the individual wells using a repeating pipette, and the microtiter plate was covered with a plate sealing film. The mixtures inside the wells were mixed with a benchtop plate shaker for about 30 s. The setup was then incubated for 1 h at room temperature (23-25 ^{o}C). After incubation, the contents inside the wells of each plate strip were removed and washed 3 times with 250 μL of Abraxis® 1X washing buffer solution (the original 5X Abraxis® washing solution was diluted at 1:5 with deionized water) using a microplate washer. The remaining buffer attached inside the wells after the final washing step was further removed by patting the plate dry on a stack of paper towels. One hundred microliters of Abraxis® color solution (substrate solution) was added to the wells, and incubated in the dark for 30 min at room temperature. One hundred microliters of Abraxis® stop solution (diluted sulfuric acid, H_2SO_4) was added to the wells in the same sequence as for the color solution. The whole plate was incubated at room temperature for 5 min before the absorbance at 450 nm was read using a Thermo Scientific® Multiskan® FC microplate photometer. The color intensity (pale yellow) formed was inversely proportional to the concentration of brevetoxins present in a sample. The toxin concentrations (μg per 100 g of shellfish tissues) in the wells were measured according to the standard curve constructed in each run using the Abraxis® PbTx-3 (dihydroBTX-B) standard solution (eight PbTx-3 concentrations: 0-2.0 ng/mL) provided by the kit. The linear calibration curve (%B/B_0 against log PbTx-3 concentrations) was established by dividing the mean absorbance value for each standard by the mean absorbance of 0 ng/mL Abraxis® PbTx-3 standard solution (Zero Standard). Samples were considered negative if the mean concentration value was lower than 0.01 ng/mL of Abraxis® PbTx-3 standard (Standard 1), while samples showing a higher concentration than 2.0 ng/mL of Abraxis® PbTx-3 standard (Standard 7) were further diluted to obtain more accurate values.

For the NSP Marbionc® ELISA kit, 0.5 mL of shellfish sample extract collected from the supernatant was defatted with 1 mL of hexane, centrifuged at 10 000×g for 10 min. Sample (0.1 mL) was collected from the bottom extraction layer, diluted to 1 mL with PGT reagent (sample diluent: PBS-Tween 20 solution was mixed with 5% melted gelatin, 9:1 v:v) to form a diluted shellfish sample solution. To establish an immobilization of brevetoxins antigens on the bottom surface of the wells, 100 μL of antigen-PBS solution (RA) was added into the wells and incubated for 1 h at 20-25 °C with slow shaking motion. The plate was then washed 3 times with 350 μL of PBS. Then, 200 μL of blocking buffer (RB) was added to the wells, incubated for 30 min at 20-25 °C with slow shaking motion. The plate was finally washed 3 times with 350 μL of PBS-Tween 20 solution (PBT). For testing the brevetoxins, 100 μL of prepared dihydroBTX-B (PbTx-3) standard solution with different concentrations and diluted shellfish sample solution was added in duplicate to each well. One hundred microliters of anti-brevetoxin antibodies reagent (RC) was then added, incubated for 1 h at 20-25 °C with slow shaking motion. The plate was then washed 3 times with 350 μL of PBT. One hundred microliters of secondary antibodies (RD) was added, incubated for 1 h at 20-25 °C with slow shaking motion. The plate was finally washed 3 times with 350 μL of PBT. The remaining buffer attached inside the wells after the final washing step was further removed by patting the dry plate on a stack of paper towels. One hundred microliters of liquid substrate 3,3', 5,5'-Tetramethylbenzidine (TMB) was added and incubated in dark at 20-25 °C with slow shaking motion for 5 min. The reaction was then terminated by adding 100 μL of 1.8 mol/L sulfuric acid (H_2SO_4) to the wells. The whole plate was read with the Multiskan® FC microplate photometer at the absorbance 450 nm. The toxin concentrations (μg per 100 g of shellfish tissues) in the wells were measured according to the standard curve constructed in each run using different amounts of the commercially available dihydroBTX-B standard

dissolved in PGT reagent (eight dihydroBTX-B standard concentrations: 0-10 ng/mL in methanol, the working range was determined as within 0.3-2.0 ng/mL). The calibration curve was analyzed by a 4-parameter logistic (4-PL) model, of which a sigmoidal relationship curve was generated, which showed the mean absorbance values against analyte (dihydroBTX-B) concentrations. This model may allow optimization of accuracy and precision of the curve over a suitable calibration range.

Two competitive ELISA kits used for testing field shellfish samples were also analyzed for their sensitivity and specificity in quantitative determination of standard brevetoxins (BTX-B and dihydroBTX-B) under different shellfish matrix-diluent combinations. For statistical analysis, an unpaired Student's *t*-test was used for comparing the difference in detection rates of brevetoxins (BTX-B and dihydroBTX-B equivalents) (percentage of toxins detection) under two individual media, namely shellfish matrix-diluent and sole kit' diluent.

RESULTS

Mice Responses to Shellfish Extracts from ACS and Non-ACS Grade Anhydrous Diethyl-ether Extraction Solvents

In order to test the suitability of a particular grade (ACS and non-ACS) of anhydrous diethyl-ether for extraction of brevetoxins in shellfish samples, a simple comparative test was conducted to investigate the difference in responses of mice injected with shellfish extracts (dissolved in cottonseed oil) individually produced from two grades of solvent extraction (Table 2). Compared with the duplicate samples (e.g. Cl1, Oy1-4 and Sc1 samples) using ACS ether for extraction, shellfish extracts produced from non-ACS ether extraction caused more and earlier deaths of mice within a 6- or 24-h monitoring period with no distinct biotoxin-induced symptoms. In addition, the non-NSP toxic symptoms (N-NSPTS), such as prolonged lethargy, weakness of limbs and slow walking, were observed, revealing the existence of certain substance(s) in shellfish extracts after extraction with non-ACS diethyl-ether which caused the death of mice. Nonetheless, the antibody-based test showed that there were no detectable brevetoxins in these samples. In general, the rectal body temperature depression during the first 6-h monitoring period was greater in mice injected with shellfish extracts produced from non-ACS diethyl-ether than in those produced from the ACS diethyl-ether. On the other hand, it has been our experience that oyster extracts dissolved in either cottonseed oil or Tween-saline often decrease the rectal body temperature and sometimes cause death in mice without causing distinct NSP-induced toxic symptoms (data not shown). These oyster toxicities in the mouse bioassay may be due to the presence of fatty acid derivatives in the samples[39-40].

Toxicity Analysis and Calibration of BTX-B Standard with ICR Mice

To establish a calibration curve for the BTX-B determination in shellfish extracts, concentrations of BTX-B standard, ranging 2.5-40 μg/mL for each mouse, under various shellfish matrix media (shellfish matrix-Tween-saline) and sole Tween-saline, were intraperitoneally injected (1-mL) for toxicity analysis. Comparison of mean survival time (minutes) for mice injected with different amounts of BTX-B standard in various matrix media are summarized in Table 3. Dosages of BTX-B between 10 and 40 μg/mL elicited prominent NSP lethal dose symptoms (NSP-LD) in mice (Table 1) and caused death of mice within a 6-h monitoring period. Although all mice (100%) died with clear NSP-LD symptoms after injecting 5 μg/mL of BTX-B standard in shellfish matrix-Tween-saline media, their survival time was beyond 24 h. Only 64% of the mice died 24 h after 2.5 μg/mL of BTX-B standard was applied. The toxicity was faster and more prominent after the doses of BTX-B made up in Tween-saline were injected into the mice than after the comparative doses of BTX-B made up in shellfish matrix-Tween-saline was injected. In addition, A significant difference (Student's *t*-test, $P<0.05$) was found in survival time of mice injected with or without the same amount of BTX-B dissolved in the two different matrix media, demonstrating that the toxicity of BTX-B in mice is suppressed in the presence of shellfish matrices. The sub-lethal dosage of BTX-B should be less than 5 μg/mL in the presence of shellfish matrix-Tween-saline media or sole Tween-saline medium. Therefore, a portion of deaths in mice administrated with 2.5 μg/mL of BTX-B in shellfish matrix-Tween-saline medium might be due to other unknown toxic effect(s) of the shellfish extracts themselves in view of the fact that no death occurred (at or after day 2 observation) in mice administrated with the same dosage of BTX-B dissolved in vehicle (Tween-saline) only.

Toxicity analysis was carried out in mice through rectal body temperature monitoring (thermoregulation). The results showed that the lethal dosage of BTX-B ranging 5-40 μg/mL, in either shellfish matrix-Tween-saline or sole Tween-saline, significantly decreased the rectal body temperature to below 35 °C in the first 30 min (Figure 1). The rectal body temperature of the most mice decreased to below 30 °C at 1 h except for the 5 μg/mL dosage in shellfish matrix-Tween-saline media. Excluding this dosage, the decreasing trend of mice rectal body temperature in both media exhibited a similar moving pattern within the 6-h monitoring period until death of mice. In addition, it should be noted that no significant difference (Student's *t*-test; $P>0.05$) was found in mice rectal body temperatures (31-33 °C) at 0.5-h for those dosages, even at the highest dosage of BTX-B applied (40 μg/mL). Thermoregulatory responses in mice to lethal dosage of BTX-B were similar when the toxin was dissolved in either shellfish matrix-Tween-saline or sole Tween-saline. The BTX-B toxicity to mice was significantly suppressed in shellfish matrix-Tween-saline media at the dose lower than 5 μg of BTX-B/mL.

The mice injected with NSP-positive shellfish samples dissolved in Tween-saline died within 1.5 h (Table 3). The NSP symptoms (lethal dose) appeared conspicuously within 30 min. With reference to the survival time of different amounts of BTX-B spiked in shellfish matrices, the amounts of total toxins in the positive samples were estimated as 250-400 μg/100 g of shellfish tissues or above. In addition, it is of interested to note that one duplicated sample Cl2(MN) extract dissolved in traditional vehicle

Table 2. Comparative Test between Mice Injected with Shellfish Extracts Dissolved in Cotton-seed Oil from Two Grades (Non-ACS: Solvent A; ACS: Solvents B and C) of Diethyl-ether(Anhydrous) Extraction Solvents and Antibody-based Test on Some of the Samples with Abraxis® Kit

Shellfish Sample	Diethyl-ether Solvent		Mice Survival Time (Min)	Temp. (°C) 0-h	Temp. (°C) 0.5-h	Mean Temp. (°C) between 1-h and 6-h	Abraxis®Results (μg/100 g of Shellfish Tissues)
Cl1 (*n*=5)[a]	A	Mean±S.E.M.	139±20.7	37.3±0.2	-	27.5±0.3	-
Cl1 (*n*=4)[a]	B (ACS)	Mean±S.E.M.	528±147	39.1±0.3	32.7±0.2	27.5?0.3	
Mu1 (*n*=6)[a]	A	Mean±S.E.M.	737±25.6	37.8±5.4	-	32.4±2.9	ND
Mu3 (*n*=2)	B (ACS)	Mean±S.E.M.	No death	39.3±0.2	36.1±1.0	37.8±0.6	-
Mu3 (*n*=2)[b]	B (ACS)	Mean±S.E.M.	No death	39.3±0.3	35.0±0.3	37.1±0.3	-
Oy1 (*n*=4)[a]	A	Mean±S.E.M.	440±60.9	37.5±0.2	34.6±0.0	26.6±1.6	ND
Oy1 (*n*=12)	B (ACS)	Mean±S.E.M.	No death	37.8±0.2	35.8±0.8	34.1±2.0	
Oy2 (*n*=8)[a]	A	Mean±S.E.M.	235±17.4	37.6±0.3	33.4±1.0	28.2±1.3	
Oy2 (*n*=4)	B (ACS)	Mean±S.E.M.	No death	38.1±0.1	33.0±0.3	26.0±0.2	ND
Oy2 (*n*=2)	C (ACS)	Mean±S.E.M.	No death	-	-	-	
Oy3 (*n*=5)[a]	A	Mean±S.E.M.	433±74.5	38.3±0.2	33.8±0.2	26.9±0.5	ND
Oy3 (*n*=4)	C (ACS)	Mean±S.E.M.	No death	38.9±0.1	32.8±0.4	28.2±0.8	
Oy4 (*n*=7)[a]	A	Mean±S.E.M.	286±21.4	37.6±0.2	-	29.1±0.9	-
Oy4 (*n*=4)	B (ACS)	Mean±S.E.M.	No death	38.2±0.2	32.7±0.3	31.0±2.4	
Sc1 (*n*=6)[a]	A	Mean±S.E.M.	644±45.0	37.6±0.2	-	32.6±1.8	ND
Sc1 (*n*=2)	B (ACS)	Mean±S.E.M.	No death	38.3±0.3	34.4±0.9	36.9±0.3	
Sc2 (*n*=8)	A	Mean±S.E.M.	No death	37.5±0.2	-	35.0±0.9	ND
Sc2 (*n*=4)	B (ACS)	Mean±S.E.M.	No death	39.0±0.3	38.3±0.1	38.3±0.1	

Note. ND: not detectable; diethyl-ether (anhydrous) B and C used were analytical reagent grade ACS and reagent grade ACS, respectively. [a]: non-NSP toxic symptoms were observed such as prolonged lethargy, weakness of limbs and walking slowly. [b]: 5% Tween 60/0.9% saline vehicle was used to dissolve the shellfish extracts instead of cottonseed oil.

Table 3. Comparison of Mean Survival Time (minutes) of Mice Administrated with Standard BTX-B and Field NSP Samples in Different Delivery Vehicles

Amount of BTX-B per mL (Mice Tested: *n*)	**40 μg (3)**	**25 μg (6)**	**20 μg (4)**	**15 μg (3)**	**10 μg[b] (15)**	**5 μg (17)**	**2.5 μg (11)**
Mice symptoms	NSP(LD)	NSP(LD)	NSP(LD)	NSP(LD)	NSP(LD)	NSP(LD)	NSP(SLD)[c]
Delivery vehicle: Shellfish matrix in Tw-Sa	Cl1 and mixSc1Sc2	Cl2, Sc1 and Oy1	Cl2 and Oy2	Cl3, Oy1 and mixCl2-3Sc1	Cl2-4, Mu1, Sc2-3, Oy2, mixOy1Oy2 and mixCl2-4Mu1Sc3	Cl3-4, Oy2 and mixCl1-4Oy2	Cl1, Mu1, Oy1, Sc1and mixCl3-4Mu1 Sc1
Mean survival time (min)±S.E.M.	30.4±3.1	87.5±9.7	95.5±13.1	190±46.6	212.3±14.1	1845±101	1926±292[d]
Amount of BTX-B per mL (Mice Tested: *n*)	**-**	**25 μg (2)**	**20 μg (6)**	**-**	**10 μg (8)**	**5 μg (4)**	**2.5 μg (6)**
Mice symptoms	-	NSP(LD)	NSP(LD)	-	NSP(LD)	NSP(LD)	NSP(SLD)[##]
Delivery vehicle:	-	Tw-Sa	Tw-Sa	-	Tw-Sa	Tw-Sa	Tw-Sa
Mean survival time (min)±S.E.M.	-	62.6±6.7	76.5±5.2[e]	-	122±9.3[e]	161±24.4	>24 h[f]
Field samples[a] (Mice Tested: *n*)	**Cl1(MN) (4)**	**Cl2(MN) (3)**	**Cl2(MN) (3)**	**Cl3(MN) (3)**	**Co1(MN)vis (2)**	**-**	**-**
Mice symptoms	NSP(LD)	NSP(LD)	NSP(LD)	NSP(LD)	NSP(LD)	-	-
Delivery vehicle:	Tw-Sa	Tw-Sa	Cso	Tw-Sa	Tw-Sa	-	-
Mean survival time (min)±S.E.M.	58.9±4.3	39.5±4.8	746±147	81.6±3.1	27.2±2.3	-	-

Note. Cso: cottonseed oil; Tw-Sa: Tween-saline (5% Tween 60/0.9% saline). Cl: clams; Co: conches; Mu: mussels; Oy; oysters; Sc: Scallops. NSP(LD): NSP (lethal dose) symptoms; NSP(SLD): NSP (sub-lethal dose) symptoms. [a]: Field samples were provided by courtesy of Mote Marine Laboratory (Sarasota, Florida, USA). [b]: 2 extractions were performed individually by ACS anhydrous diethyl-ether B and C to produce shellfish extracts for the BTX-B spiked test. [c]: No NSP symptom was observed after 24 h. [d]: 7 out of 11 mice died after experiment. [e]: For 20 μg of BTX-B dissolved in 1 mL cottonseed oil, the mean survival time (mean±SEM) of mice was 185±36.2 min (*n*=8) with typical symptoms of NSP(LD) in mice. For 10 μg of PbTx-2 dissolved in 1 mL cottonseed oil, no mice died after 24 h monitoring and no significant typical sign of NSP(LD) was found in mice besides labor breathing, slight hind leg paralysis and weakness. [f]: 4 of the mice were not tested after 24 h (day 2), but the others remained alive for up to 72 h and all mice looked healthy at day 2 observation.

(cottonseed oil) showed a longer survival time than that dissolved in Tween-saline, though the NSP (lethal dose) symptoms were also demonstrated. According to the two toxin spiked tests, the toxicity of 10 and 20 μg BTX-B/mL dissolved in traditional vehicle cottonseed oil was lower than that of corresponding BTX-B dosages dissolved in Tween-saline. Moreover, the mice NSP symptoms after these dosages were moderate, in particular the dosage 10 μg of BTX-B/mL in cottonseed oil medium. Of the 8 NSP-positive control samples injected into the mice, 4 samples significantly decreased the rectal body temperature within 1.5 h until death (Figure 1). Similar to those samples with lethal doses of BTX-B individually dissolved in shellfish matrix-Tween-saline and sole Tween-saline media, the rectal body temperature was 31 °C and 33 °C, respectively, at 0.5-h for mice injected with these positive samples. In addition, a similar moving trend of temperature decrease was observed after application of the lethal dose of BTX-B dissolved in shellfish matrix- Tween-saline and sole Tween-saline media.

The sub-lethal dose (2.5 μg/mL) of BTX-B dissolved in both shellfish matrix-Tween-saline and Tween-saline media, did not decrease the rectal body temperature to below 33 °C as was observed when the other lethal dosages were applied (Figure

2), though the temperature maintained at below 33 °C after 3 h. Moreover, the rectal body temperature was finally elevated to almost normal (above 34 °C and 38 °C for 2.5 μg/mL of BTX-B in shellfish matrix-Tween-saline and sole Tween-saline media) in the next day (24-h). On the other hand, the other 4 NSP-positive control samples showed no [Co2(MN) or Co3(MN)], but only slightly decreased rectal body temperature at 0.5-h with mild NSP sub-lethal dose symptoms during the 6-h monitoring period. Therefore, the NSP-positive samples were estimated to have NSP toxins at the level below 2.5 μg of BTX-B equivalent in 1 mL of sample injection. The estimated BTX-B equivalent levels in the shellfish were less than 25 μg of BTX-B equivalent per 100 g of shellfish tissues.

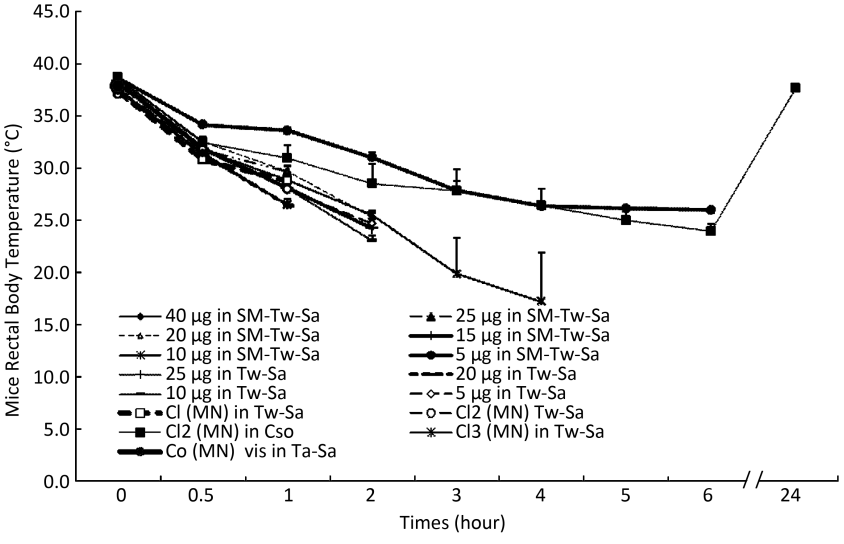

Figure 1. Changes of rectal body temperature in mice after intraperitoneal injection of (1) different doses of BTX-B standards (μg/mouse) dissolved in 1 mL of shellfish matrix-Tween- saline and solely Tween-saline, and (2) 4 NSP-positive control shellfish samples (lethal) extracts dissolved in 1 mL of Tween-saline vehicle (one of Cl2(MN) sample extracts was dissolved in cottonseed oil). Data are represented by mean±SEM. Cso: cottonseed oil; SM: Shellfish matrices; Tw-Sa: Tween-saline (5% Tween 60/0.9% saline); No. of mice and details of SM used for each test: refer to Table 3.

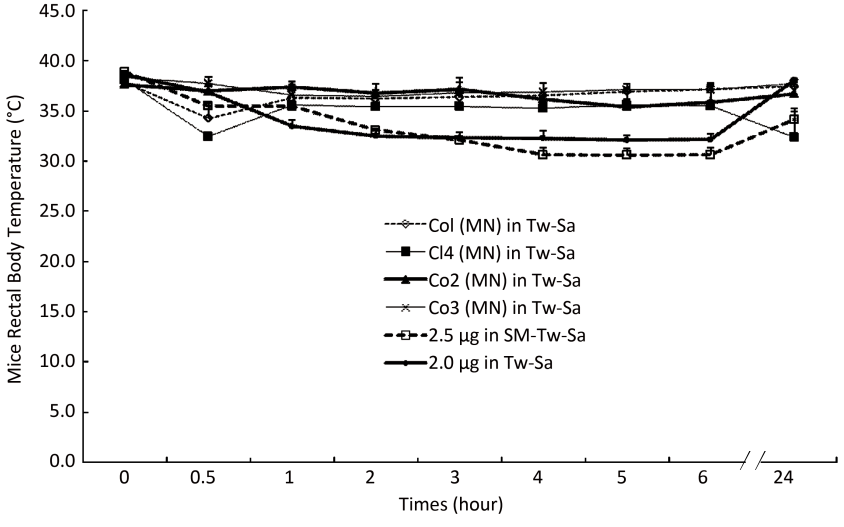

Figure 2. Changes of rectal body temperature in mice after intraperitoneal injection of (1) BTX-B standard (2.5 μg/mouse) dissolved in 1-mL of shellfish matrix-Tween-saline and solely Tween-saline, and (2) 4 NSP-positive control shellfish samples (sublethal) extracts dissolved in 1 mL of Tween-saline vehicle. Data are represented by mean±SEM. SM: Shellfish matrices; Tw-Sa: Tween-saline (5% Tween 60/0.9% saline); No. of mice used for Co1(MN), Co2(MN) and Cl4(MN) tests=4; Co3(MN) test=2. No. of mice and details of SM used for 2.5 μg tests: refer to Table 3.

Calibration of BTX-B standard in ICR mice was established by plotting a linear regression line using the data from BTX-B spiked sample extracts (Table 3). LD_{50} values for BTX-B (ICR mice) in shellfish matrix-Tween-saline and sole Tween-saline media were identified on y-intercept using corresponding linear correlation between (dose) (ordinate) and (dose/time) (abscissa) (Figure 3). For BTX-B dissolved in Tween-saline, the LD_{50} value was 250 μg/kg (5.0 μg per 20 g ICR mouse) in female ICR mice. On the other hand, the LD_{50} value of BTX-B was identified as 455 μg/kg (9.1 μg per 20 g ICR mouse) within 36 h in female ICR mice in shellfish matrix-Tween-saline media (excluding oyster extracts samples). The correlation coefficient (r) for the line was 0.92. It should be noted, however, that including oyster matrix samples slightly increase the LD_{50} value to 10 μg per 20 g ICR mouse with mild deterioration of the correlation coefficient (from r=0.92 to r=0.89) and the slope of the regression line.

By plotting a calibration curve using Log (MU) versus Log (1/T) based on the LD_{50} values identified, equations were established for determining an approximate BTX-B equivalent level in shellfish matrix-Tween-saline and sole Tween-saline media (Figure 4). The results showed that there was a great

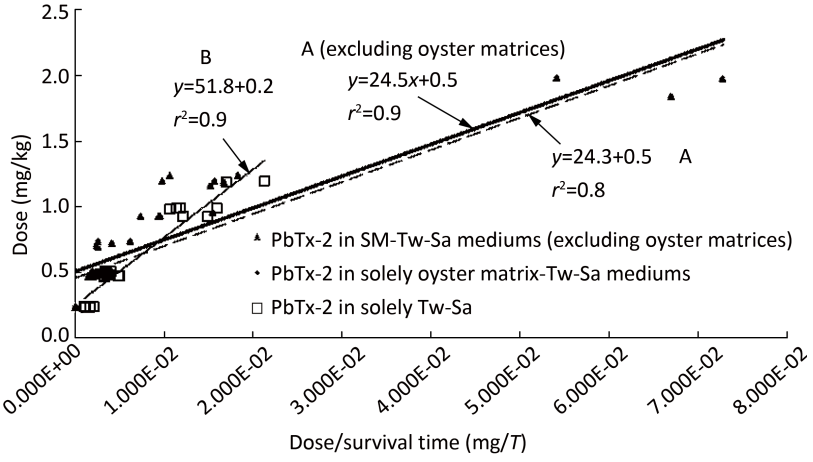

Figure 3. Dose (mg/kg) against dose/survival time (mg/T) for mice injected with different amounts of BTX-B (PbTx-2) dissolved in (A) shellfish matrix-Tween-saline and (B) solely Tween-saline media. SM-Tw-Sa: shellfish matrix-Tween-saline; Tw-Sa: Tween-saline**.**

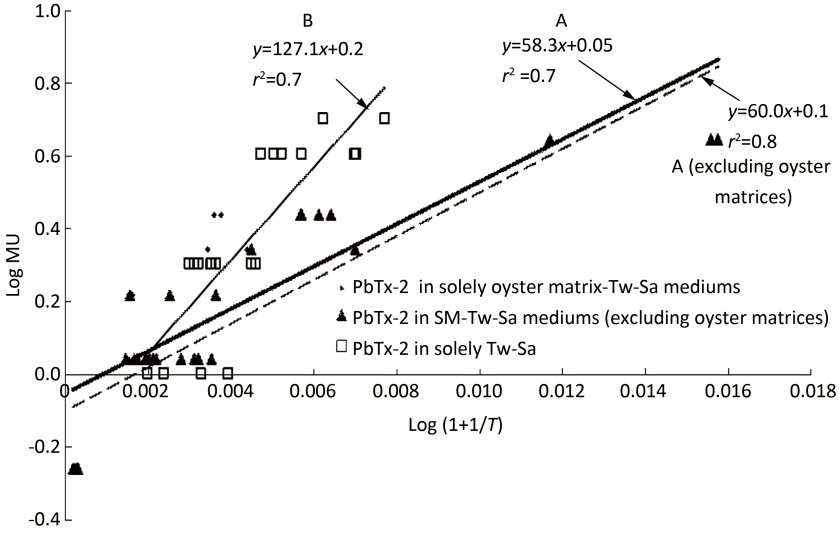

Figure 4. Log (MU) against log (1+1/T) for mice injected with different amounts of BTX-B (PbTx-2) dissolved in (A) shellfish matrix-Tween-saline and (B) solely Tween-saline media. SM-Tw-Sa: shellfish matrix-Tween-saline; Tw-Sa: Tween-saline; MU: mouse unit; T: mice survival time (minutes).

discrepancy in survival time between the mice injected with BTX-B in shellfish matrix-Tween-saline media and those injected with BTX-B in sole Tween-saline medium. The toxicity of BTX-B dissolved in sole Tween-saline medium was unambiguously higher than that dissolved in shellfish matrix-Tween-saline media. In addition, the presence of oyster matrices prominently weakened the value of correlation coefficient (*r*) from 0.88 to 0.82. As a result, the established equation y=60.0x-0.1 was adopted as an appropriate formula to evaluate the toxicity levels of NSP toxins (BTX-B equivalent level) in shellfish samples in which considerations were taken into account on the general matrix effect(s) found in various shellfish matrix-Tween-saline media and the relatively high matrix effects produced by oyster samples were also excluded.

Evaluation on Performance of NSP ELISA Kits in Detecting BTX-B in Shellfish Samples

PbTx-3 (dihydroBTX-B) standards provided by Abraxis® were used to calibrate for the test in each run. The stability of the standards (8 concentration points between 0.010-2.0 ng/mL including 0 ng/mL) was investigated by plotting a standard curve using B/B_0 (%) versus the corresponding toxin concentrations [Log concentration ($\times 10^{-2}$ ng/mL)] (Figure 5). No significant fluctuation of B/B_0 (%) values was observed over the 34 runs using different lots of Abraxis® ELISA kit in different times. The correlation coefficient value (*r*) was 0.995.

The performance (percentage of brevetoxins detection) of Abraxis® ELISA kit on the determination of commercially available dihydroBTX-B (Abraxis® and CalBiochem®) standards and BTX-B standards (CalBiochem®, Marbionc®, and Latoxan®) individually in solely diluent and shellfish matrix-diluent media are shown in Table 4. For the dihydroBTX-B in solely diluent, the overall coefficient correlation (*r*) and percentage of brevetoxins detection were 0.99 and 81.3%±6.1%, respectively (Figure 6). For the dihydroBTX-B in different shellfish matrix-diluent media (clams, mussels, oysters and scallops), the overall coefficient correlation (*r*) and percentage of brevetoxins detection were 0.99 and 113±8.5%, respectively (Figure 6, Table 4). It should be noted that the correlation of expected and calculated values of dihydroBTX-B began to diverge at the toxin concentrations of 0.5 ng/mL and 1.0 ng/mL spiked individually into both media. Although the results showed that the Abraxis® ELISA kit demonstrated a similar response to dihydroBTX-B in both media, a significant difference was found in percentages of dihydroBTX-B detection between them (Student's *t*-test, P<0.05). Moreover, the detection rate of toxin started to deteriorate from 0.25 ng/mL of dihydroBTX-B (Figure 6). The good linear correlation between expected and calculated values of dihydroBTX-B tended to decrease at or above the concentration 0.25 ng/mL. An unacceptable detection rate of dihydroBTX-B (<50%) was observed when 1 ng/ml of dihydroBTX-B spiked individually in both media. For testing the percentages of BTX-B detection with Abraxis® kit, two concentrations (0.16 and 0.20 ng/mL) were adopted and the detection rate of the toxin in solely diluent and shellfish matrix-diluent media were about 73%-79%. On the other hand, although a significant difference was found between expected and calculated values for dihydroBTX-B detection in shellfish-diluent medium and in solely Abraxis® diluents (Student's *t*-test, P<0.05), the detection rate of dihydroBTX-B in both media was still comparable. According to this study, the acceptable detection range of dihydroBTX-B for Abraxis® kit was 0.015-0.25 ng/mL in solely diluent and shellfish matrix-diluent media.

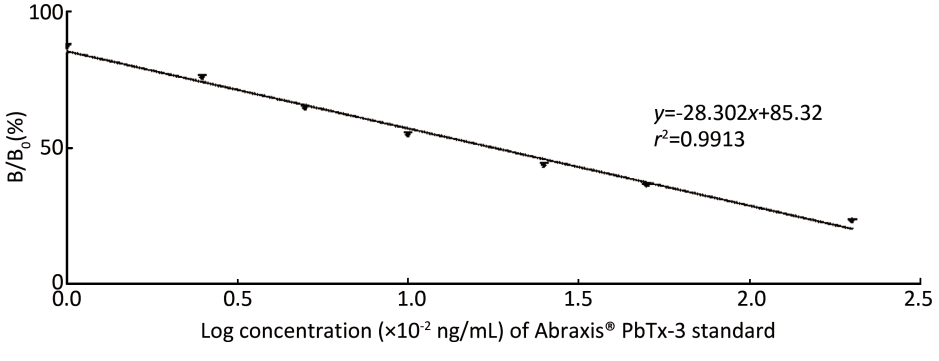

Figure 5. B/B_0 (%) against Log concentration ($\times 10^{-2}$ ng/mL) of Abraxis® PbTx-3 (dihydro BTX-B) standard over 34 runs in different times.

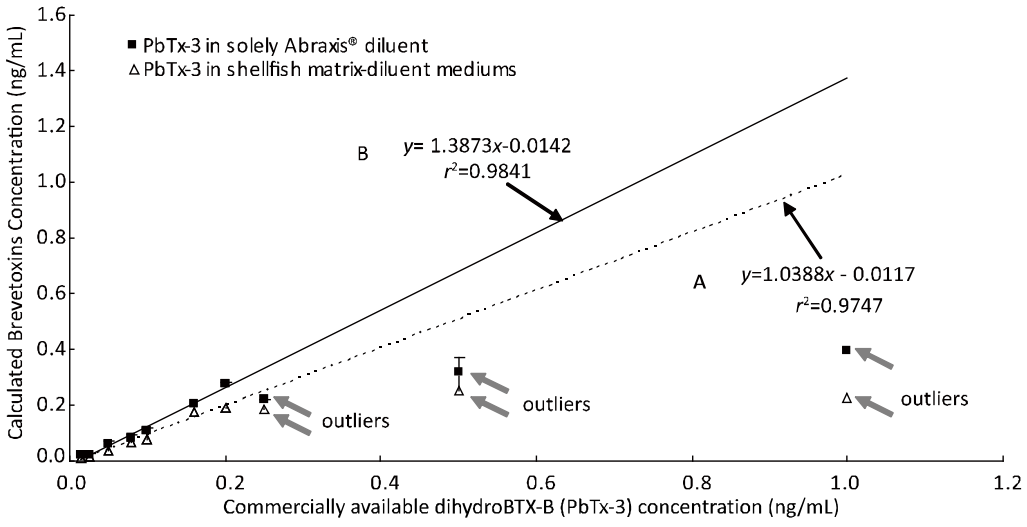

Figure 6. Abraxis® kit for the measurement of different concentrations of a commercially available dihydroBTX-B (PbTx-3) standard (standard C) dissolved in (A) shellfish matrix-diluent media and (B) solely Abraxis® diluent. Data are represented as mean±SEM. No. of tests (*n*) performed on the kit: 0.015-0.2 ng/mL of PbTx-3 standards, *n*=4; 0.25-1.0 ng/mL of PbTx-3 standards, *n*=2. Shellfish matrix-diluent mediums included clams-, mussels-, oysters- and scallops-matrix.

Table 4. Brevetoxins (BTX-B and DihydroBTX-B) Detection Rate between Two Antibody-based Assays Using Different Brands of Standard Toxins Dissolved in Shellfish Matrix-diluent Media and Solely ELISA Diluents (%)

ELISA Kit	**Marbionc®**			**Abraxis®**		**Marbionc®**		**Abraxis®**		**Abraxis®**	
Matrix	PGT			Shellfish-Diluent[a]	Diluent	Shellfish-PGT[b]	PGT	Shellfish-Diluent[c]	Diluent	Shellfish-Diluent[d]	Diluent
Brand tested	A	B	C	C		C		C		Abraxis® standard	
Brevetoxin (Concentrations, ng/mL)	BTX-B (1.25, 0.63, 0.31)			BTX-B (0.2, 0.16)		dihydroBTX-B (2, 1, 0.5) for Shellfish-PGT (1.25, 0.63, 0.31) for PGT		dihydroBTX-B (0.015-0.25) 8 points		PbTx-3 (dihydroBTX-B) (0.10, 0.20) for Shellfish-Diluent (0.10, 0.20, 0.25) for Diluent	
% of toxins detection (mean±S.E.M.)	106±6.6	125±15	88±5.2	73.2±4.5	79.1±0.3	86±1.3	87±1.8	113±8.5	81.3±6.1	130±12.0	103±5.3
No. of tests (*n*)	3	3	3	2	2	12	3	8	8	7	10
P values[e]	>0.05	<0.05	>0.05	<0.05	<0.05	>0.05[f]	>0.05	>0.05	<0.05	<0.05	>0.05

Note. The confidence value (CV) of the results from both ELISA kits was <15% except for one dihydroBTX-B spiked (2 ng/mL) oyster sample extract tested by Marbionc® assay. Acceptance criterion set for the percentage of detection: 80%-120%. PGT: phosphate buffer saline (PBS)-Tween 20 solution was mixed with 5% melted gelatin; 9:1 v:v. (diluent of Marbionc® ELISA kit). [a]: clams-, oysters- and scallops-matrix mediums. [b]: scallops-matrix mediums only. [c]: clams-, mussels-, oysters- and scallops-matrix mediums. [d]: clams-, oysters- and scallops-matrix mediums. [e]: Student's *t*-test *P* values were obtained by comparing the calculated toxin concentrations with their corresponding expected concentrations under different matrix mediums. [f]: except spiked shellfish extracts with concentrations of dihydroBTX-B at 2.0 ng/mL.

With reference to Marbionc® study showing the detection rate of dihydroBTX-B in shellfish matrix-PGT and sole PGT media (Table 4), the average toxin detection rate in both media was about 85%-87%, which could meet the acceptance criteria (over 80%). However, no significant difference was found between expected and calculated levels of dihydroBTX-B (Student's *t*-test, *P*>0.05). The toxin detection rate (88%) was also comparable with that using a same brand of BTX-B.

In addition, no significant difference was observed between the expected and calculated levels of BTX-B measured with Marbionc® kit. Nevertheless, the BTX-B detection rate by Marbionc® using two other brands (brands A and B) of BTX-B instead showed a prominent difference in toxin detection even with the same concentration of toxin. It should be noted that a significant difference was also found when the results between expected and calculated levels from brand B BTX-B were analyzed using the three-point check by Marbionc® kit (Student's *t*-test, $P<0.05$) because there was a prominently elevated toxin dete ction rate (154%) calculated at the expected concentration of 0.31 ng/mL. In general, the BTX-B detection rate with Marbionc® kit was over 80% for toxin in PGT (sample diluent) medium. According to this study, the acceptable detection range of dihydroBTX-B for Marbionc® kit was 0.31-2.0 ng/mL both in solely PGT and in shellfish matrix-PGT media.

Comparison of NSP-Positive Control Results between ICR Mouse Bioassay and Antibody-based Detection Analysis

Samples from NSP-positive control showing high brevetoxin levels in our ICR (female) mouse bioassay also showed high brevetoxin levels by both ELISA studies. A summary of results determined by the three detection assays is shown in Table 5.

The BTX-B equivalent results identified from our ICR mouse bioassay demonstrated that the lethal dose determined from the NSP-positive samples matched well with those of the two antibody-based assays except for a Co1(MN) vis sample (an outlier). Comparative analysis between both ELISA kits showed comparable results except for the first test in Co1(MN) vis sample (479 μg of dihydroBTX-B equivalent/100 g) determined with Marbionc® kit. This sample was an outlier in the inter-comparison test (Table 5). In general, the NSP-positive control samples with a low dihydroBTX-B equivalent level in the two antibody-based assays could not be identified to have brevetoxins (BTX-B equivalent) in ICR mouse bioassay, though some NSP sub-lethal dose (NSP(SLD)) symptoms were observed in certain samples such as Co1(MN), Co2(MN) and Co3(MN) (Table 5). Inter-comparison analysis showed that the correlation coefficient value (*r*) except for the outlier Co1(MN) vis was ≥0.96 (mean: $r=0.98$) (Student's *t*-test, $P>0.05$). From our ICR mouse bioassay, the BTX-B equivalent levels below 25 μg per 100 g of shellfish tissues were measurable. These NSP-positive (sub-lethal doses) samples, previously determined as "not detectable" by our ICR mouse bioassay, were identified as below 25 μg BTX-B equivalent level per 100 g of shellfish tissues with the two ELISA kits.

Table 5. NSP Detection Results between NSP ICR Mouse Bioassay and Two Antibody-based Assays (Abraxis® and Marbionc® ELISA Kits)

NSP-positive Control Samples	NSP ICR Mouse Bioassay[a] (μg of BTX-B Equivalent/100g±S.E.M.) (No. of Mice Used=*n*)	Abraxis® ELISA (μg of DihydroBTX-B Equivalent/100g)[b]	Marbionc® ELISA (μg of DihydroBTX-B Equivalent/100g)[b]
Cl1(MN)	204±16 (*n*=4)	328	279; 234[d]
Cl2(MN)	348±52 (*n*=3)	393	316; 344[d]
Cl3(MN)	151±4.3 (*n*=3)	203	226; 216[d]
Cl4(MN)	ND (*n*=4)	11.4	14.7
Co1(MN)	ND[#] (*n*=4)	8.6	10.7
Co2(MN)	ND[#] (*n*=3)	9.5	6.6, 6.7[d]
Co3(MN)	ND[c] (*n*=2)	4.0	3.7, 3.8[d]
Co1(MN)vis	652±116 (*n*=2)	234	479; 197[d]
Mu[e]	ND (*n*=2)	5.7	10.5

Note. The coefficient of variations (CVs) of the results obtained in each run from both ELISA kits was <15%. Correlation coefficient value (excluding an outlier Co1(MN)vis) obtained by inter-comparison between assays was ≥0.96 (mean: $r=0.98$, Student's *t*-tests, $P>0.05$). ND: not detectable. [a]: NSP toxins extraction was performed using solvent B anhydrous diethyl-ether (ACS). [b]: the results were calculated according to the dihydroBTX-B (PbTx-3) standard curves generated by the kits in each run. [c]: some NSP(SLD) symptoms were observed in these samples. Co1(MN): slight tail convulsions, hind leg paralysis and walking imbalance; Co2(MN): slight labored breathing; Co3(MN): hind leg paralysis. [d]: the results were obtained from another ELISA test on different days using shellfish extracts produced from second methanol extraction. [e]: a mussel sample collected in Hong Kong market.

DISCUSSION

Although alternative technologies are available for the detection and identification of brevetoxins[41-42], mouse bioassay is still widely accepted as a gold standard method for screening brevetoxins in shellfish samples[19].

In the first part of this study, the specific solvent requirement recommended in official NSP mouse bioassay method confirmed that an analytical reagent grade ACS of anhydrous diethyl-ether was necessary for brevetoxin extraction. Shellfish extracts by ACS anhydrous diethyl-ether with either analytical reagent ACS or reagent grade ACS exerted a similar matrix effect on mice. It is of interest that harmful effects were observed on mice injected with those extracts from non-ACS anhydrous diethyl-ether. Deviation in mice responses on extracts from the two grades of solvents might be due to the difference in purity of the solvents. It should be noted that certain specifications of diethyl-ether solvent such as alcohol, carbonyl and color conditions are not specified for the non-ACS ether. In addition, the amount of a common antioxidant (BHT, ~5 ppm) in non-ACS diethyl-ether was about 5-10 times higher than that of the two ACS ether solvents, suggesting that more BHT is needed to prevent deterioration of the solvent during storage. Moreover, the amount of all impurities would be very significant after having been concentrated originally from 200 mL of diethyl-ether with 50 g shellfish extracts to a final 5 ml of shellfish matrix-Tween-saline mixture. As a result, ACS anhydrous diethyl-ether with good quality (e.g. reagent/analytical grade or above) is necessary for extraction of NSP toxins from shellfish in order to exclude unnecessary substance(s) in the sample extracts affecting the health of mice.

The two antibody-based assays used in this study showed that they were able to identify and quantify the dihydroBTX-B (in shellfish matrix-diluent media) within certain concentration ranges (0.015-0.25 ng/mL for Abraxis®; 0.31-2.0 ng/mL for Marbionc®) with an acceptable toxins detection rate (80%-120%). In addition, in general, both kits could also quantify over 70% of BTX-B from the expected toxin concentrations. It should be noted, however, that the manufacturer claims on Abraxis® kit instruction sheet indicate that there would be about 100% and 102% of toxin detection (cross-reactivity) with dihydroBTX-B (PbTx-3) and BTX-B (PbTx-2), respectively. Our results showed deviation from those values in the serial dilution tests, which might be due to the effect of the shellfish matrix and/or the purities of the commercially available toxin standards. According to the two concentrations tests (0.10 and 0.20 ng/mL) using Abraxis® PbTx-3 standard dissolved in shellfish matrix-diluent media (Table 4), the presence of shellfish matrices significantly affected the toxins detection rate when compared with Abraxis® PbTx-3 in the Abraxis® diluent medium in the absence of shellfish matrix. Moreover, there was a discrepancy in toxins detection rate with the Abraxis kit while testing different concentrations of a commercially available dihydroBTX-B standard (Standard C) made up in a shellfish matrix-diluent and when it was made up in the Abraxis® diluent media in the absence of shellfish matrix. In addition, commercially available BTX-B and dihydroBTX-B standards used in this study were not certified reference materials (CRMs). There is no common reference/guarantee for the purities of standards, and accurate calibration on the toxin standards is not achievable in laboratory. As a result, traceability to a common reference for comparison is impossible. Nevertheless, our study showed no significant deviation in the results of each dihydroBTX-B concentration from the calibration curves produced by both kits in each run. The confidence values (CVs) of all results obtained from both ELISA kits were all within an acceptable limit of less than 15%, except for one dihydroBTX-B spiked (2 ng/mL) oyster sample extract tested by Marbionc® assay. In general, brevetoxins (dihydroBTX-B equivalent) levels (0.68-11.3 μg/100 g, 2.88 μg/100 g and 18 μg/100 g of shellfish tissues) could be measured in shellfish with Abraxis® kit and Marbionc® kit respectively, which are far below the NSP regulatory limit of 80 μg/100 g of shellfish tissues. As a result, both kits could be used as a first-line screening assay for identifying BTX-B and dihydroBTX-B (mainly dihydroBTX-B equivalent level) in field shellfish samples. Recently, Naar et al. (2002)[29] also demonstrated that an antibody-based assay could be successfully applied in quantifying brevetoxins in shellfish samples with a detection limit at 2 μg/100 g of shellfish tissues. Nevertheless, further studies are still needed to elucidate the cross-reactivity levels of various BTXs and their metabolites in antibody-based assays and investigate the difference in potencies of these toxins between ELISA and mouse bioassay. It should be noted that brevetoxins exist primarily in the form of different

metabolites due to species-specific variations of metabolism in shellfish[8-10,14,40,44]. BTX-B present in NSP causative microalgae is rapidly metabolized in shellfish into its metabolites such as BTX-B2−5 and dihydroBTX-B[8-10,13-14]. The common BTXs in shellfish include BTX-B2 (majority of BTX in shellfish) and dihydroBTX-B with an almost same potency in immunoassay analysis[14,33,39,45]. The most potent BTX-B4, though accounts for two-thirds of the total mouse toxicity[13,33], has not been recognized well by ELISA[33]. As a result, there would be a risk of missing toxic BTX-B4 and other fatty acids form toxins in shellfish by ELISA test. In a public health perspective, use of ELISA for first line screening of shellfish is necessary to identify the most common and abundant BTXs (potentially containing dihydroBTX-B/BTX-B2) for subsequent confirmatory analysis of total BTX toxicity by the developed NSP mouse bioassay. Although both official NSP mouse bioassay and our bioassay used BTX-B as a standard to evaluate NSP BTX-B equivalent levels in contaminated shellfish, it is recommended that dihydroBTX-B should be a more appropriate calibration standard instead of BTX-B for reflecting NSP toxicity levels in shellfish and for comparative analysis between mouse bioassay and ELISA. Ishida et al.[9-10] also suggested that dihydroBTX-B, a relatively persistent BTX metabolite in shellfish, can be used as a suitable biomarker for monitoring NSP toxicity in shellfish because BTX-B is an unstable toxin due to the structure of beta unsaturated aldehyde which is rapidly reduced to its metabolites in shellfish tissues. According to *in vivo* analysis of both BTX-B and dihhydroBTX-B in mice[7,46], their acute toxicity levels are rather similar. Moreover, toxicity responses in mice are comparable based on our test (Table 1). BTX-B, therefore, is deemed suitable as a calibration standard for NSP mouse bioassay we developed in this study.

According to the BTX-B toxicity in ICR mice, there was a significant difference in toxicity responses between mice injected with BTX-B in different shellfish matrix media and solely Tween-saline vehicle (5% Tween 60/0.9% saline). The survival time of mice injected with BTX-B in solely Tween-saline vehicle was shorter than that of those injected with BTX-B in shellfish matrices. Therefore, shellfish matrices could affect the toxicity expression of BTX-B in ICR mice. The survival time of mice injected with 10 and 20 μg of BTX-B in 1 mL of cottonseed oil (traditional vehicle used in APHA NSP mouse bioassay) was longer than that of those injected with the same amount of BTX-B in both shellfish matrix-Tween-saline and solely Tween-saline media. Hannah et al.[27] reported that there is no apparent difference in toxic effects as measured in mice injected with shellfish extracts dissolved in solely vehicle (1% Tween 60/0.85% saline) and in those injected with shellfish extracts dissolved in cottonseed oil. In the present study, a statistically significant difference was observed in both media (Student's *t*-test, $P<0.05$). In addition, it is obvious that cottonseed oil is more viscous than Tween-saline vehicles. As a result, considerable caution should be taken in selecting an appropriate delivery vehicle for NSP mouse bioassay. The use of Tween-saline vehicle can make the application of i.p. injection more efficient and smooth without clogging problem as encountered at the injection needle opening throughout this study. Moreover, the shellfish extracts could be dissolved quite well with Tween-saline vehicle.

By investigating the thermoregulatory mechanism in female ICR mice after administration of BTX-B in both shellfish matrix-Tween-saline and solely Tween-saline media, it should be noted that there was a similar pattern of temperature decrease between them within the 6-h monitoring period. In addition, the temperatures at 0.5-h were 31-33 °C between BTX-B dosages of 10 and 40 μg/mL for each ICR mouse in both matrix media. Therefore, the attainment of that temperature range at 0.5 h could be considered as an additional symptom for testing high levels of BTX-B equivalent toxicity (10-40 μg/mL in shellfish matrix-Tween-saline medium; i.e. 100-400 μg/100 g of shellfish tissues) in female ICR mouse bioassay. For the ICR mice died at over 30 h with NSP (lethal dose) symptoms, a BTX-B equivalent level could be set at 2.5-5.0 μg/mL (i.e. 25-50 μg/100 g of shellfish tissues) in shellfish matrix-Tween-saline media, while a level of "less than 2.5 μg/mL (i.e. less than 25 μg/100 g of shellfish tissues)" for samples in shellfish matrix-Tween-saline media could be assigned when the NSP (sublethal dose) symptoms were observed in mice. In order to adopt an appropriate calibration curve to evaluate the levels of NSP toxins (BTX-B equivalent level) in field shellfish samples, the effects of shellfish matrices on toxicity expression in experimental animals should be considered as a vital factor for influencing the accuracy of the calculated levels. In our study, shellfish matrices showed a significant effect on the toxicity responses in ICR mice as compared with those injected with BTX-B in solely vehicle medium.

According to the LD_{50} curves shown in Figure 3, 9.1 μg/20 g in female ICR mice was used as LD_{50} for BTX-B in shellfish matrix-Tween-saline media (excluding the oyster matrices). It should be noted that oyster matrices in the calculation slightly elevated the LD_{50} (i.e. 10 μg/20 g female ICR mice) with changes in the equation slope and decrease in the correlation coefficient values (*r*) from 0.92 to 0.89. Based on the study on survival time of mice administrated standard BTX-B, the injection of 10 μg/mL BTX-B, in either shellfish matrix-Tween-saline or solely Tween-saline vehicle caused 100% death of female ICR mice with prominent NSP (LD) symptoms. Therefore, LD_{50} should be less than this value. The present LD_{50} value is about 2-fold higher in ICR mice than in Swiss-Webster strain mice[24,26], which may be due to the difference in strains of mice used in both assays. Moreover, Selwood et al.[47] also found that toxicity of brevetoxins is more severe in mice after fasting action. In this study, no fasting procedure was applied in mice throughout the tests. On the other hand, in our experience, the weights (i.e. the amounts) of oysters extract obtained after diethyl-ether extraction were often higher than those of other shellfish extracts (data not shown), and the shellfish matrix-Tween-saline mixtures made from oyster samples were more viscous than other shellfish matrix-Tween-saline mixtures produced from other shellfish samples. Therefore, it is plausible that the matrix effects on oyster samples are stronger and significant. In order to consider the errors from the matrix effects, and preserve the accuracy of calculation under general shellfish matrices, an equation $y=60.0x-0.1$ produced from mice injected with BTX-B under general shellfish matrices dissolved in Tween-saline (excluding those oyster samples) was appropriately adopted to evaluate BTX-B equivalent levels in field shellfish samples using female ICR mice as an experimental model in the bioassay. For the field oyster samples, the NSP-positive values could also be assayed using the same formula, provided that the NSP (lethal/sublehtal dose) symptoms of mice were clearly observed. It is likely, however, that the actual BTX-B equivalent levels in oyster samples were underestimated.

By comparing the NSP-positive control results between our established NSP female ICR mouse bioassay and the two antibody-based tests, there was a strong correlation in results between the 3 groups of individual analysis. Marbionc® test was performed by an independent operator in order to prevent subjectivity in the ELISA analysis. Overall, the NSP-positive controls with a high brevetoxin level (BTX-B equivalent level) in ICR mouse bioassay also showed a high brevetoxin level (dihydroBTX-B equivalent level) by both ELISA studies except for a conch sample Co1(MN)vis (an outlier). The matrix effect of the shellfish (visceral parts) extracts might be one of the key factors affecting the ELISA results. Furthermore, experimental error(s) produced during sampling procedures and/or individual extraction techniques in each shellfish sample for the two ELISA tests might also be possible. Since extraction of shellfish with diethyl-ether is inefficient to extract all brevetoxin derivatives, in particular polar metabolites in oysters[44], results from the ELISAs and/or bioassay suggest possible underestimation. Based on the test results between these 3 detection methods, ICR (female) mouse bioassay can achieve the same results as the two ELISA analyses, and can, therefore, be used as an acceptable confirmatory assay for toxicity evaluation (BTX-B equivalent level) in shellfish samples. It has been shown that mouse bioassay is well correlated with the competitive ELISA for toxicity evaluation in shellfish samples[42,48-49].

Brevetoxins are heat- and acid-stable lipidic molecules that can remain toxic after normal cooking or food preparation procedures. They tend to accumulate in fatty tissues of mammals not easily metabolized or excreted from the body. Despite the disadvantages of the mouse bioassay such as poor sensitivity, slow and low throughput, its key application in NSP monitoring program of shellfish farms and markets is necessary to further confirm the violation of regulatory toxicity levels identified from other chemical-, structural- and/or functional-based analysis. Although no fatal human case of NSP has yet to be identified, there is still a potential risk for susceptible groups such as children, elderly and those in developing areas where there is little means for diagnosing this poisoning. Establishment of reliable screening assays coupled with a promising mouse bioassay for further confirmatory test is an essential element under a shellfish monitoring program in order to protect a community against this biotoxin poisoning. In addition, use of chemical analysis by LCMS can additionally provide a legally defensible confirmation of particular brevetoxins and their related metabolites in shellfish sample(s) according to their retention time and ions chromatograms. The cytotoxicity of neuroblastoma and receptor binding assays, pharmacology-based methods based on

brevetoxins actions on voltage-gated sodium channels, are also good supporting alternatives of screening assays for brevetoxins in shellfish[41-42]. These analytical and sensitive *in vitro* methods can sufficiently replace the mouse bioassay in the future.

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DECLARATIONS

The authors declare that there are no conflicts of interest in this study. All commercial products, trade names or materials mentioned in this article do not represent any suggestion, approval or endorsement for use by authors' department, division and organization. All experimental mice used in the bioassay were sacrificed after NSP test in compliance with Code of Practice Care and Use of Animals for Experimental Purposes, Agriculture, Fisheries and Conservation Department of Hong Kong, China.

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