Development of an Enzyme-Linked Immunosorbent Assay for the Detection of Ciguatoxin in Fish Tissue Using Chicken Immunoglobulin Y

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A sandwich enzyme-linked immunosorbent assay was developed to detect ciguatoxin (CTX) in fish tissue. The assay utilizes two antibodies, chicken immunoglobulin Y specific to the ABCD domain of CTX and a mouse monoclonal immunoglobulin G-hor-

seradish peroxidase conjugate specific to the JKLM domain of CTX. The sensitivity, working range, cross reactivity, accuracy, precision, and reproducibility were examined. J. Clin. Lab. Anal. 22:239–245, 2008. © 2008 Wiley-Liss, Inc.

Key words: ELISA; marine toxins; ciguatera fish poisoning; ciguatera

INTRODUCTION

Ciguatoxins (CTXs) (Fig. 1a) are responsible for ciguatera fish poisoning, a seafood intoxication caused by ingestion of specific contaminated species of tropical and subtropical reef fishes. Produced by the marine benthic dinoflagellate *Gambierdiscus toxicus*, CTXs are biomagnified through the food chain, ultimately resulting in human and mammalian illness. They represent a family of complex, lipid-soluble, highly oxygenated cyclic polyether compounds that are stable, colorless, odorless, and cannot be inactivated by cooking or freezing (1). Ciguatera fish poisoning is characterized by neurological, gastrointestinal, and cardiovascular manifestations as early as 4 hr after ingestion of ciguatoxic fish, and poses a health risk at levels above 0.1 ppb or ~100 pg/ml (2). In the past, such toxins have been confined to seafood-dependent populations in island or coastal regions; however, with increased tourism, international fishing trade, and the importation of various reef fish by the seafood industry, there have been increasing reports of marine toxin illnesses in inland parts of the world. An estimated 50,000 cases of ciguatera fish poisoning are reported each year worldwide (3), although that figure is likely a low conservative estimate owing in part to misdiagnosis and underreporting.

Current assays to screen for CTX include: intraperitoneal injection of mice with crude extracts of fish (4,5); sodium channel assays such as the neuroblastoma (NB) assay and fluorometric NB-based assay (6,7); a mem-

brane immunobead assay (MIA) developed by Hokama et al. (8), which is a specific qualitative detection method based on antibody technology; and analytical methods such as high-performance liquid chromatography and liquid chromatography-mass spectroscopy (2,9). Drawbacks to the currently available screening methods include nonspecificity, costly routine screening, and questionable reliability for yielding quantitative results. Although enzyme-linked immunoassays specific for CTX or synthetic fragments of the toxin have been recently reported in the literature (10,11), none have been shown to adequately screen fish extracts, nor have any utilized chicken Immunoglobulin Y (IgY) as an immunoanalytic tool.

Enzyme-linked immunosorbent assay (ELISA) is a sensitive, specific method that has been used to successfully screen for small molecules in seawater, fish tissue extracts, and mammalian body fluids. To counter some of the disadvantages of mammalian antibodies, avian egg yolk antibody has been identified as a convenient, noninvasive, inexpensive source of antibodies (12,13). IgY is a counterpart of mammalian

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Fig. 1. (a) Ciguatera molecule, (b) ABCD fragment, and (c) JKLM fragment.

immunoglobulin G (IgG) present in the egg yolk of aves, reptiles, and amphibians (14), differing in molecular weight (IgG = 150 kDa, IgY = 180 kDa) from IgG. Like IgG, IgY has two heavy chains and two light chains; however, IgY has four constant regions compared with three for IgG (15). Chicken IgY antibodies have been used in recent years to develop ELISA assays in a variety of disciplines and are a promising new tool in assay development.

This study details a sensitive and specific sandwich ELISA developed for the detection of CTX in fish tissue that was preliminarily introduced as a poster abstract at the Experimental Biology Conference in Spring 2007 (16). The assay uses an IgY immunoglobulin produced in chickens immunized with the ABCD fragment of CTX (anti-CTX ABCD) and a mouse anti-CTX IgG that recognizes the JKLM fragment of CTX (anti-CTX JKLM). Figure 1a–c shows the molecular structure of the whole molecule and the fragments targeted by the antibodies.

MATERIALS AND METHODS

Antibody Preparation

Mouse anti-CTX JKLM was produced as previously described (17), and maintained as a hybridoma cell line in tissue culture flasks (Costar, Corning, NY) in Dulbecco's modified Eagle medium (Sigma Chemical

Company, St. Louis, MO), kept at 37°C with 5% CO₂. Antibody was fractionated and affinity purified using Protein A column chromatography for use in assay development. This antibody was conjugated to horseradish peroxidase (HRP) using the EZ Link Activated Peroxidase Kit (anti-CTX JKLM-HRP) (Pierce Chemical Company, Rockford, IL).

The ABCD fragment of the CTX molecule was chemically synthesized and generously provided by Professor Minoru Isobe (Nagoya University, Japan). Five hundred micrograms of the fragment was conjugated to 50 mg of human serum albumin using an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma Chemical Company, St. Louis, MO) mediated conjugation as described by Hokama et al. (18). The resulting immunogen conjugate was sent to Aves Labs, Inc. (Tigard, OR) for injection into chickens. The IgY fraction containing the ABCD fragment of CTX, as well as preimmune chicken IgY, was collected and stored at 4°C for use in ELISA development.

CTX Extracts and Other Marine Toxins

Toxic fish samples previously implicated in ciguatera poisoning were obtained from the Hawaii Department of Health (DOH). Fish were submitted from patients with clinical symptoms verified by an attending physi-

cian or the DOH Epidemiology Branch staff and tissues were extracted and partially purified according to the method of Kimura et al. (5). The CTX-positive extract was evaluated for the presence of CTX using the MIA, mouse toxicity, NB, and guinea pig atrial assays (4). Based on the NB assay and inhibition studies using the MIA technique, the CTX-positive extract was calculated to contain approximately 0.32 ng of CTX per mg of extract (6,8). The same extraction and purification procedures for processing fish extract were followed for a deep water pelagic fish that is not expected to not harbor CTX (Pacific blue marlin, *Makaira nigricans*) to serve as a nontoxic control for use in experiments.

Purified brevetoxin-3 (PbTx-3), okadaic acid, and domoic acid were purchased from Calbiochem (San Diego, CA) for cross reactivity studies.

Sandwich ELISA for Detection of CTX

A sandwich ELISA was developed in the following manner: 96-well ELISA plates (Costar, Corning, NY) were coated with 0.25 mg/ml anti-CTX ABCD antibody in phosphate-buffered saline (PBS), pH 7.4, 100 μ l/well, and incubated overnight at 4°C. Nonspecific binding sites were blocked with a milk-free blocking solution, Superblock (Pierce Chemical Company, Rockford, IL), 200 μ l/well for 5 min followed by three washes with PBS wash buffer, pH 7.4. Partially purified CTX-positive extract was diluted in 20% methanol:80% PBS buffer at concentrations ranging from the methanol:PBS buffer alone as a control and 0.004–0.5 mg/ml. One hundred microliters of the control and standards were added to each well in quadruplicate, and plates were incubated for 1 hr at room temperature followed by three washes with PBS to remove excess antigen. Anti-CTX JKLM-HRP monoclonal antibody (30 μ g/ml in PBS, pH 7.4) was added to each well, 100 μ l/well, and incubated for 1 hr at room temperature followed by six thorough washes with PBS to remove residual antibody. Bound antibodies were visualized by the addition of 100 μ l/well *o*-phenylenediamine (Sigma Chemical Company, St. Louis, MO). Reaction was allowed to develop for 5 min before being stopped by the addition of 50 μ l/well 1 N sulfuric acid. Absorbance was measured at 490 nm using a TiterTek microplate reader (BioTek Instruments, Winooski, VT). Results are expressed as relative absorbance units.

The specificity of the assay was further assessed using a competitive ELISA method in which the anti-CTX ABCD antibody was coated onto the microplate overnight and 1 mg/ml of CTX-positive extract in 20% methanol:80% PBS buffer and a buffer control were applied as described in the previous section and incubated for 1 hr. Simultaneously in a separate test

tube, CTX-positive extract at 0.032, 0.125, and 0.5 mg/ml was added to 30 μ g/ml of anti-CTX JKLM-HRP antibody in PBS and incubated for 1 hr at room temperature. A control was also performed in which the antibody was incubated alone without any added extract. After washing the microplate with PBS, 100 μ l per well of each of the above extract-antibody mixtures was transferred to the CTX-treated ELISA plate and incubated for 1 hr. The assay proceeded as described previously, and signal obtained in the presence of the inhibitor and the absence of the inhibitor was plotted showing percent inhibition against the free CTX concentration. All experiments were performed in triplicate and mean values were obtained.

NB Cell Assay

The NB cell assay was modified after the method of Manger et al. (6). Mouse NB cells (neuro-2a, CCL-131, ATCC, Manassas, VA) were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM glutamine, 1 mM sodium pyruvate, 50 μ g/l streptomycin, and 50 units/ml penicillin. Cultures were maintained at 37°C in a humidified 5% CO₂, 95% air atmosphere. NB cells from stock culture were seeded in complete media in 96-well microtiter plates (Costar) at a concentration of 1.5×10^5 cells/ml in 100 μ l volumes per well and maintained at 24 hr at 37°C. After 24 hr, 10 μ l each of 10 mM ouabain and 1 mM veratridine and dilutions of both partially purified CTX-positive and nontoxic fish extracts were added to wells in triplicate. Control wells of ouabain/veratridine (OV) alone and untreated controls received media to make up for volume differences. The plates were incubated at 37°C for 16–20 hr for the detection of sodium channel inhibition. End-point assessment using (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum was carried out as described previously (6), and color end point was read at a wavelength of 490 nm using a TiterTek microplate reader. Reagents were purchased from Sigma Chemical unless otherwise indicated. Data were analyzed to obtain the mean and standard deviation of triplicate wells.

Statistical Analysis

Statistical calculations were performed using Microsoft Excel and GraphPad Prism 5.0 software (San Diego, CA).

RESULTS

ELISA Development, Optimization, and Validation

Several different ELISA platforms and antibody combinations were tested, and a checkerboard titration was performed to determine the optimal reagent

concentrations. Because CTX may be susceptible to conformation changes in a purely aqueous solution, various ratios were tested in order to determine the optimal solvent to buffer ratio for use in the assay, which was found to be 20% methanol:80% PBS. In order to show that the anti-CTX ABCD antibody was reactive when compared with other coating materials, bovine serum albumin (BSA), preimmune chicken IgY, and anti-CTX ABCD chicken IgY at 0.25 mg/ml in PBS were used to coat the microplate and the assay was performed as described. BSA and preimmune IgY showed partial reactivity as a coating substrate; however, statistical two-tailed unpaired *t*-test analysis showed that the anti-CTX ABCD IgY antibody is the optimal solid-phase antibody in the ELISA as indicated by a higher relative absorbance when detecting CTX-positive extract at levels above 0.016 mg/ml ($P < 0.05$).

The NB cell assay, a commonly accepted method of assessing the activity of marine toxins on sodium channels *in vitro* using the agonistic effects of ouabain and veratridine, was performed on both the partially purified CTX-positive extract and a nontoxic marlin extract at the following concentrations: 0, 0.032, 0.065, 0.125, 0.25, and 0.5 mg/ml. The results of the assay show that the CTX-positive extract induces cytotoxicity in a dose response manner as expected for extracts containing CTX when compared with an OV control sample, whereas the nontoxic marlin extract shows no cytotoxicity when compared with the OV control. Comparing CTX-positive extract with the OV control, one-way analysis of variance (ANOVA) indicates statistical significance at $P < 0.0001$ and Tukey's multiple comparison test indicates significance ($P < 0.05$) for extract concentrations from 0.125 to 0.5 mg/ml, equivalent to approximately 40–160 pg of CTX, whereas the nontoxic marlin extract shows no statistical variation between samples when compared with the control ($P = 0.1299$, Tukey's multiple comparison test shows that $P > 0.05$ for all data points).

As a further validation of the reactivity between the CTX-positive extract and the anti-CTX JKLM antibody, an inhibition study was performed wherein anti-CTX ABCD antibody was coated to the plate overnight and 1 mg/ml of CTX-positive extract was applied. The anti-CTX JKLM-HRP antibody was preincubated for 1 hr in a separate test tube with varying concentrations of the CTX-positive extract. A control was also performed in which the antibody was incubated alone without any added extract. Antibody and extract mixtures were added to the assay plates and tested for reactivity. Results show a 46% reduction when blocking with 0.032 mg/ml (10 pg CTX equivalent), a 52% reduction in reactivity at 0.125 mg/ml (40 pg CTX equivalent), and a 69% reduction at 0.5 mg/ml (160 pg

Fig. 2. Competitive inhibition showing that CTX-positive extract blocks the reactivity of anti-CTX JKLM-HRP when incubated together for 1 hr before the assay. Each data point represents the mean of triplicate determination with vertical bars indicating + standard deviation. Statistical significance indicating inhibition when compared with the zero control is represented by * ($P < 0.05$), and statistical significance when comparing the 0.032 and 0.5 mg/ml results is represented by ** ($P < 0.05$). CTX, ciguatoxin; HRP, horseradish peroxidase.

CTX equivalent) blocking concentrations with respect to the control (Fig. 2) indicating that the extract is binding and effectively blocking the antibody from reacting with available sites in the assay. One-way ANOVA indicates that each of the concentrations assayed is significantly different ($P < 0.0001$) when compared with the control, and one-tailed unpaired *t*-test shows statistical significance between the 0.032 and 0.5 mg/ml data points ($P < 0.0295$).

Limit of Detection and Range

Figure 3 shows dose response linear regression log-log transformed ELISA results for the partially purified CTX-positive extract ($P = 0.004$, $r^2 = 0.89$, slope = 0.353 ± 0.050) and nontoxic marlin fish extract ($P = 0.400$, $r^2 = 0.12$, slope = 0.013 ± 0.015), demonstrating the specific affinity of the ELISA for CTX in extract. When statistically evaluated using a two-tailed *t*-test in comparison with the nontoxic marlin, the lower limit of detection was determined to be 0.016 mg/ml of CTX-positive extract ($P = 0.0003$) or approximately 5 pg/ml of equivalent CTX per mg of extract. The upper limit of the working range was found to be 2 mg/ml of CTX-positive extract or approximately 640 pg/ml of equivalent CTX per mg of extract. CTX equivalents are based on previous work shown in Hokama et al. (8).

Precision

The repeatability (intra-assay variation) and the reproducibility (inter-assay variation) of the sandwich ELISA were

Fig. 3. Log-log transformed ELISA results for partially purified CTX-positive extract and nontoxic marlin extract. Each data point represents the mean of triplicate determination. Statistical significance indicating the lower detection limit of CTX-positive extract when compared with the nontoxic marlin extract is represented by * ($P=0.0003$). ELISA, enzyme-linked immunosorbent assay; CTX, ciguatoxin.

performed using concentrations of CTX-positive extract at 0.016, 0.032, 0.065, 0.125, 0.25, and 0.5 mg/ml, plus a control consisting of the 20% methanol:80% PBS buffer alone. The coefficient of variation for the intra-assay using said concentrations ($n=10$) was found to be in the range of 4–22%, whereas the inter-assay ($n=8$) showed a range of 5–24%, with most values coming in at approximately 10%. Tables 1 and 2 summarize the data.

Recovery

Various concentrations of CTX-positive extract, from 0.016 to 0.5 mg/ml, were spiked to a nontoxic marlin fish sample (weight = approximately 0.2 g) characterized as negative for CTX, acetone extracted, and subsequently assayed using the ELISA for recovery of sample. Percent recovery ranges from 75 to 106% (Fig. 4). The data were analyzed using two-tailed paired t -tests to assess significance between the recovery and the control at each concentration. The results indicate no significant differences between the control and the percent recovered ($P>0.05$) at each of the data points assayed.

Cross Reactivity

Purified marine toxins brevetoxin-3, okadaic acid, and domoic acid were spiked into 0.25 mg/ml of nontoxic marlin extract and tested for cross reactivity with the ELISA as described in the concentration range of 0.065–20 ng/ml. Although brevetoxin-3 showed a slight reaction, statistically significant cross reactivity was not observed for any of these marine toxins in the specified range.

DISCUSSION

Enzyme immunoassays are used widely in both clinical and research fields because they are rapid, simple, accurate, cost effective, and specific. This assay

TABLE 1. ELISA Intra-Assay Coefficient of Variation

CTX-positive extract in mg/ml	Number of determinations	Mean value (o.d.)	Standard deviation (o.d.)	CV (%)
0 Control	10	0.039	0.009	22.3
0.008	70	0.071	0.011	15.5
0.016	10	0.082	0.008	9.55
0.032	10	0.145	0.013	9.24
0.065	10	0.256	0.016	6.31
0.125	10	0.305	0.013	4.35
0.25	10	0.316	0.020	6.45
0.50	10	0.383	0.022	5.80

Intra-assay coefficients of variation for data points ranging from the buffer alone as a control to 0.50 mg/ml of CTX-positive extract. The mean and the standard deviations for each data point from ten separate replicates performed on the same day are shown. ELISA, enzyme-linked immunosorbent assay; CTX, ciguatoxin.

TABLE 2. ELISA Inter-Assay Coefficient of Variation

CTX-positive extract in mg/ml	Number of determinations	Mean value (o.d.)	Standard deviation (o.d.)	CV (%)
0 Control	8	0.058	0.013	22.9
0.008	8	0.094	0.020	21.4
0.016	8	0.171	0.042	24.6
0.032	8	0.286	0.043	15.3
0.065	8	0.321	0.026	8.18
0.125	8	0.365	0.031	8.72
0.25	8	0.375	0.033	8.85
0.50	8	0.338	0.018	5.42

Inter-assay coefficients of variation for data points ranging from the buffer alone as a control to 0.50 mg/ml of CTX-positive extract. The mean and the standard deviations for each data point from eight separate replicates performed on different days are shown. ELISA, enzyme-linked immunosorbent assay; CTX, ciguatoxin.

represents an improvement on current detection methods in that it detects low levels of CTX in fish extras with no significant cross reactivity with structurally similar polyethers and other marine toxins, including brevetoxin-3 that shares a similar molecular backbone to CTX, and okadaic acid, which has shown cross reactivity with antibody-based assays previously (8,19).

The ELISA, which takes approximately 4 hr to complete and can be performed using 96-well assay plates to accommodate a large number of samples, is capable of detecting between 5 pg/ml and 2 mg/ml of CTX according to CTX equivalent data. Comparing the detection limits of other common methods of CTX detection, the NB cell assay is capable of detecting as low as 1 pg/ml of purified PCTX-1 and 3 pg/ml of CTX3C after 7 hr of incubation (20), but the method is not conducive to large-scale rapid screening nor is it

Fig. 4. Percent recovery of DOH CTX-positive extract after spiking a nontoxic marlin fish sample. Data represent the mean of triplicate evaluations and vertical bars indicate $\pm$ standard deviation. The results indicate no significant differences between the control and the percent recovered ($P > 0.05$) at each of the data points assayed. DOH, Department of Health; CTX, ciguatoxin.

necessarily specific to CTX. The mouse bioassay can detect CTX as low as 5 ng/ml depending on the congener and extraction techniques used (21,22); however, its drawbacks include lack of specificity and require the maintenance of live animals. The MIA shows a reasonable limit of detection for CTX at approximately 32 pg/g tissue of CTX (8), whereas the liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry application (2) has been reported to detect levels of CTX equivalent to 40 ng/kg of PCTX-1 in fish flesh and 100 ng/kg of CCTX-1 in fish flesh.

A limiting factor in the MIA (8) is the background noise or nonspecific binding that has the potential to produce false-positive results. The development of a two-site ELISA circumvents this issue while maintaining sensitivity and low cross reactivity. The ability of the described ELISA to detect low toxin levels is likely owing to several factors, including the use of a synthetic fragment to create antibodies, which may have reduced the lethality of the toxin such that an appropriate immune response in chickens could be achieved, and the selection of purified IgY as the immobilized antibody in the ELISA, which can reduce background interference. This specificity may be a disadvantage, however, in screening for other congeners of the CTX molecule not present in the CTX-positive extract used to develop this assay. More than 12 structurally related variants have been identified in the Pacific (21–27), differing from those found in the Caribbean (28,29) and Indian Oceans (9), none of which have been specifically evaluated using this ELISA to date. However, the intent is to screen fish

from these regions using several tiered methods to determine the applicability of the assay to Pacific congeners not represented in the CTX-positive extract as well as major toxic CTX congeners found in the Caribbean and Indian Oceans.

Because reliable, purified CTX was not available in a sufficient quantity to serve as standards, the assay was optimized for the detection of CTX using a partially purified CTX-positive extract that had been previously characterized using a number of bioassays accepted in the marine toxin research community, including the mouse toxicity and guinea pig atrial trial. The use of such extracts in assay development is routine when purified or synthetic toxin is scarce (2,8,17,30–32), and simple recalibration can be performed following purification and characterization of new extracts to maintain assay consistency. Additional steps were taken to standardize the assay, including the extraction of a deep water pelagic fish that is not expected to not harbor CTX, *M. nigricans*, to rule out the fact that the lipid matrix or the extraction solvents might affect the assay result. The lack of availability of reliable purified CTX standards to further validate the assay may affect the interpretation of the results with respect to the exact sensitivity of the assay and what true levels of CTX present in the partially purified fish extract are being detected as opposed to estimates of equivalence, and leaves the door open for further studies to be done toward improvement of the method. However, because the ELISA was developed using CTX-positive fish extract rather than purified compounds, the transition into screening wild caught fish samples is more streamlined, as the assay has already been optimized for use with the complex matrix of lipids and other background that accompanies testing fish tissues.

In conclusion, the development of this ELISA demonstrates the usefulness of chicken IgY as an immunoanalytic tool and provides a sensitive, specific methodology to test for CTX in fish extracts, which has broad implications for consumers and commercial fishing operations alike.

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REFERENCES

1. Murata M, Legrand AM, Ishibashi Y, Fukui M, Yasumoto T. Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. *J Am Chem Soc* 1990;112:4380–4386.
2. Lewis RJ, Jones A, Vernoux JP. HPLC/tandem electrospray mass spectrometry for the determination of sub-ppb levels of Pacific and

- Caribbean ciguatoxins in crude extracts of fish. *Anal Chem* 1999;71:247–250.
3. Baden DG, Fleming LE, Bean JA. 1996. Marine toxins. In: deWolff FA, editor. *Handbook of Clinical Neurology: Intoxications of the Nervous System Part II. Natural Toxins and Drugs*. Amsterdam: Elsevier. 199 p.
 4. Hokama Y, Asahina AY, Titus E, et al. Assessment of ciguateric fish in Hawaii by immunological mouse toxicity and guinea pig atrial assay. *Mem Qld Museum* 1994;34:489–496.
 5. Kimura LH, Hokama Y, Abad MA, Oyama M, Miyahara JT. Comparison of the different assays for the assessment of ciguatoxin in fish tissue: Radioimmunoassay, mouse bioassay and in vitro guinea pig atrium assay. *Toxicon* 1982;20:907–912.
 6. Manger RL, Leja LS, Lee SY, et al. Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *J AOAC Int* 1995;78:521–527.
 7. Louzao MC, Vieytes MR, Yasumoto T, Botana LM. Detection of sodium channel activators by a rapid fluorometric microplate assay. *Chem Res Toxicol* 2004;17:572–578.
 8. Hokama Y, Takenaka W, Nishimura KL, Ebesu JSM. A simple membrane immunobead assay for detecting ciguatoxin and related polyethers from human ciguatera intoxication and natural reef fishes. *J AOAC Int* 1998;84:727.
 9. Hamilton B, Hurbungs M, Jones A, Lewis RJ. Multiple ciguatoxins present in Indian Ocean reef fish. *Toxicon* 2002;40:1347–1353.
 10. Oguri H, Hirama M, Tsumuraya T, et al. Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. *J Am Chem Soc* 2003;125:7608–7612.
 11. Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M. Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. *Toxicon* 2006;48:287–294.
 12. Polson A, Von Wechmar MB, Van Regenmortel MHV. Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol Commun* 1980;9:475–493.
 13. Jensenius C, Anderson J, Hau J, Crove M, Koch C. Eggs: Conveniently packaged antibodies. *Methods for purification of yolk IgG*. *J Immunol Methods* 1981;46:63–68.
 14. Warr GW, Magor KE, Higgins DA. IgY: Clues to the origins of modern antibodies. *Immunol Today* 1995;16:392–398.
 15. Chiou V. Duck antibodies for IVD applications. URL: <http://www.devicelink.com/ivdt/archive/02/04/003.html>. 2003. Accessed November 11, 2006.
 16. Empey-Campora C, Hokama Y, Yabusaki K, Isobe M. 2007. Development and validation of an enzyme-linked immunosorbent assay (ELISA) for the detection of ciguatoxin using chicken immunoglobulin Y as an analytic tool. Poster presented at Experimental Biology Conference, Washington, DC, Spring.
 17. Hokama Y. Simplified solid-phase immunobead assay for detection of ciguatoxin and related polyethers. *J Clin Lab Anal* 1990;4:213–217.
 18. Hokama Y, Banner AH, Boylan DB. A radioimmunoassay for the detection of ciguatoxin. *Toxicon* 1997;15:317–325.
 19. Hokama Y, Hong TWP, Isobe M, Ichikawa Y, Yasumoto T. Cross reactivity of highly purified okadaic acid (OA), synthetic, spiroketal east sphere of OA and ciguatoxin. *J Clin Lab Anal* 1992;6:54–58.
 20. Hokama Y. 2004. Ciguatera fish poisoning: Features, tissue, and body effects. In: Preedy V, Watson R, editors. *Reviews in Food and Nutrition Toxicity*, Vol. 2. Florida: CRC Press. p 43.
 21. Lewis RJ, Sellin M. Multiple ciguatoxins in the flesh of fishes. *Toxicon* 1992;30:915–919.
 22. Lewis RJ, Sellin M, Poli MA, Norton RS, MacLeod JK, Sheil MM. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus* Muraenidae). *Toxicon* 1991;29:1115–1127.
 23. Murata M, Legrand AM, Ishibashi Y, Yasumoto T. Structures of ciguatoxin and its congener. *J Am Chem Soc* 1989;111:8929–8931.
 24. Lewis RJ, Holmes MJ. Origin and transfer of toxins involved in ciguatera. *Comp Biochem Physiol C* 1993;106:615–628.
 25. Satake M, Murata M, Yasumoto T. The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. *Tetrahedron Lett* 1993;34:1975–1978.
 26. Satake M, Fukui M, Legrand AM, Cruchet P, Yasumoto T. Isolation and structures of new ciguatoxin analogs, 2,3-dihydroxy-yctx3c and 51-hydroxyctx3c, accumulated in tropical reef fish. *Tetrahedron Lett* 1998;101:1197–1198.
 27. Yasumoto T. The chemistry and biological function of natural marine toxins. *Chem Rec* 2001;1:228–242.
 28. Pottier I, Vernoux JP, Jones A, Lewis RJ. Characterisation of multiple Caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (*Caranx latus*) by high-performance liquid chromatography/mass spectrometry. *Toxicon* 2002;40:929–939.
 29. Pottier I, Hamilton B, Jones A, Lewis R, Vernoux JP. Identification of slow and fast-acting toxins in highly ciguatoxic barracuda (*Sphyraene barracuda*) by HPLC/MS and radiolabelled ligand binding. *Toxicon* 2003;42:663–672.
 30. Bottein Dechraoui MY, Wang Z, Turquet J, et al. Biomonitoring of ciguatoxin exposure in mice using blood collection cards. *Toxicon* 2005;46:243–251.
 31. Bottein Dechraoui MY, Tiedeken JA, Persad R, et al. Use of two detection methods to discriminate ciguatoxins from brevetoxins: Application to great barracuda from Florida Keys. *Toxicon* 2005;46:261–270.
 32. Shimojo RY, Iwaoka WT. A rapid hemolysis assay for the detection of sodium channel-specific marine toxins. *Toxicology* 2000;154:1–7.