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Detection of Ciguatoxin in Fish Tissue Using Sandwich ELISA and Neuroblastoma Cell Bioassay

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The applicability of a new enzyme-linked immunoassay (ELISA) for detecting ciguatoxin (CTX) in fish tissue was evaluated by testing three fish species commonly implicated in ciguatera fish poisoning in Hawaii. A total of 164 individual almaco jack (*Seriola rivoliana*) and greater amberjack (*S. dumerili*) and a total of 175 individuals of the blue-spotted grouper (*Cephalopholis argus*) were caught at various locations in the Hawaiian Islands. Muscle tissue from each individual was assessed for the presence of CTX using two methods: a semi-quantitative ELISA that was recently developed for detecting picogram levels of CTX in fish extract and a neuroblastoma (NB) cell assay commonly used to screen for marine toxins in fish. Results of the tests were highly correlated, with the ELISA indicating the presence of CTX in 9.4% of all fish samples, and the NB assay indicating toxicity in 6.8% of the fish samples. We conclude that the ELISA produces reliable and accurate results that are consistent with those provided by the accepted NB assay and that the ELISA has potential for future applications in screening fish populations for CTX. J. Clin. Lab. Anal. 22:246–253, 2008.

Key words: ciguatoxin; ciguatera fish poisoning; roi; kahala; ELISA

INTRODUCTION

Ciguatera fish poisoning in humans is an intoxication resulting in characteristic gastrointestinal and neurological symptoms following the ingestion of fish containing ciguatoxin (CTX) or one of its congeners. It is the most common marine toxin poisoning worldwide, with more than 50,000 cases reported annually (1,2), and poses a health risk at levels above 0.1 ppb or ~100 pg/ml (3). The incidence rate in Hawaii is approximately 3.6 cases per 100,000 people annually (4), which is low compared with other regions in the tropics such as French Polynesia (e.g., 23,000 cases per 100,000 people (2)). In the main Hawaiian Islands (MHI) the majority of the cases are reported from leeward coastlines, in particular west Oahu, the north shore of Kauai, west Maui, and the west and northwestern coasts on the Island of Hawaii (5).

CTX is a lipophilic toxin produced by the marine dinoflagellate *Gambierdiscus toxicus* (6), which is found in marine macroalgae associated with coral reefs. CTX can be passed from one trophic level to the next, e.g., from herbivorous fish to carnivorous fish, and can thus bio-accumulate through the marine food chain (1,7,8). The toxin is heat stable, colorless, odorless, and cannot be inactivated through cooking, canning, drying, freezing, smoking, salting, or pickling (1).

The blue-spotted grouper (*Cephalopholis argus*, Bloch & Schneider), regional Hawaiian name roi, was intro-

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duced to the MHI from Moorea in 1957 with the intent to establish a new fishery (9). After an initial time-lag in population growth, roi has become the dominant large nearshore predatory reef fish in the MHI (10). However, despite the fact that roi is an economically valuable species in many of its native habitats, the envisioned fishery did not develop after roi caused a number of ciguatera fish poisoning incidents in Hawaii and was rejected by consumers (11,12). Twenty-one incidents of ciguatera from roi consumption were reported in Hawaii between 1996 and 2000, representing 17% of all reported incidents (11).

Similarly, during the 1970s, the almaco jack, *Seriola rivoliana* Valenciennes, and the greater amberjack, *S. dumerili* Risso, collectively known as kahala, were the fish most frequently associated with ciguatera fish poisoning in Hawaii, responsible for 21% of ciguatera incidents reported to the State Department of Health between 1975 and 1981 (5). Wild-caught kahala were subsequently excluded from sale in commercial markets owing to the risk of ciguatera fish poisoning. Historical data on kahala catch and sales in Hawaii over time are summarized in Figure 1 and illustrate the sharp decline in marketability of this species concurrent with associated outbreaks of ciguatera fish poisoning from the mid-1970s to the present.

Several biological, immunological, and chemical methods are available for detecting CTX in fish tissue, including a traditional mouse bioassay, radioligand binding, high-performance liquid chromatography, and mass spectrometry. However, none of these tests are well suited for routine large-scale screening of fish samples. This includes the neuroblastoma (NB) cell assay, which is an established method for the detection of marine toxins including CTXs in crude seafood extracts using directed cytotoxicity by recording sensitive changes in the sodium channel (13). It provides accurate results with a low number of false positives, but is not suited to large-scale screenings because of the complicated methodology nor is the test specific to CTX. In contrast, the membrane immunoassay developed by Hokama et al. (14) is a qualitative field assay used to detect CTX, although it has been shown to cross react with other marine toxins (15), and has the potential to yield a high false-positive rate depending on the fat content of the fish (16). To address the shortcomings of the existing assays, a sandwich enzyme-linked immunoassay (ELISA) was recently developed (17) to detect whether CTX in partially purified fish extract is semi-quantifiable and has shown promise in eliminating some of the problems observed when using other methods.

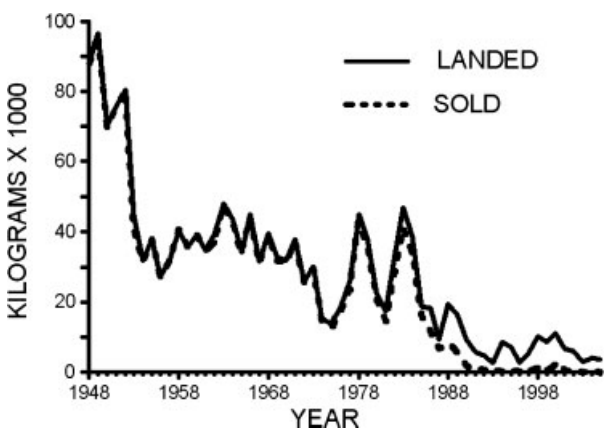

Fig. 1. Historical data for kahala caught and sold in Hawaii from 1948 to 2002. *Source*: Division of Aquatic Resources.

The objective of this study was to evaluate the potential of the newly developed ELISA as a screening tool for CTX. For this purpose, its accuracy and precision were validated by testing the presence of CTX in fish species commonly implicated in ciguatera fish poisoning in Hawaii using both the ELISA and the accepted NB assay, followed by statistical comparison of results obtained using the two methods.

MATERIALS AND METHODS

Fish Collection

Roi individuals were caught by spearfishing on SCUBA at various sites along the west (Kona) coast of the Island of Hawaii ($n = 93$; standard length (SL): 28.8 ± 5.6 cm, mean $\pm$ SD) and at sites around the Island of Oahu ($n = 82$; SL: 26.5 ± 5.3 cm) in July and August 2003. Kahala were obtained by hook and line from the Penguin Banks in the MHI ($n = 83$; fork length (FL): 75.1 ± 9.5 cm) and from various sites in the northwestern Hawaiian Islands ($n = 81$; FL: 73.5 ± 14.4 cm) between December 2006 and August 2007.

Sample Preparation

Fish were stored on ice during transport back to a laboratory, where a 2.5-cm $\times$ 2.5-cm cube of muscle tissue without the skin was excised from the left side or the head region of each fish and stored at -20°C until lipid extraction could be performed. For lipid extraction, the muscle tissue was placed in a clean test tube and soaked in 1 ml of 100% acetone per 2.5 g of fish tissue overnight. The acetone extract was then transferred to a clean, tared test tube, blown down using a laboratory air source, and weighed. The extract was resuspended in 100% methanol at a concentration of 10 mg/ml and stored at 4°C for further CTX analysis.

Positive controls for CTX for use in the assay validation were obtained by extracting the lipids of toxic fish samples previously implicated in ciguatera fish

poisoning as determined by the Hawaii Department of Health (DOH). Fish were submitted from patients with clinical symptoms verified by an attending physician or the DOH Epidemiology Branch staff. Tissue from these samples was extracted and partially purified according to the method of Kimura et al. (18). The CTX-positive extract was evaluated for the presence of CTX using mouse toxicity, NB, and guinea pig atrial assays (19). Based on the NB assay and inhibition studies, the CTX-positive extract was calculated to contain approximately 0.32 ng of CTX per mg of extract (13,14).

To rule out the lipid matrix or the extraction solvents potential to affect the ELISA or NB assay results, the same extraction procedures were followed for a deep water pelagic fish that is not expected to harbor CTX, Pacific blue marlin (*Makaira nigricans* Lacepède), purchased from a commercial market, referred to herein as nontoxic fish extract.

ELISA for CTX Detection

A sandwich ELISA was recently developed to detect picogram levels of CTX in partially purified fish extract (17). This method was applied to evaluate the lipid extracts from each wild-caught fish sample for CTX. Briefly, 96-well ELISA plates (Costar, Corning, NY) were coated with anti-CTX capture antibody specific to the ABCD region of the CTX molecule in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Nonspecific binding sites were blocked with a milk-free blocking solution, Superblock (Pierce Chemical Company, Rockford, IL), followed by three washes with PBS. The lipid extract from each fish sample was diluted in 20% methanol:80% PBS buffer for use in the assay at 1 mg/ml. A volume of 100 µl of each sample was added to wells in triplicate, and plates were incubated for 1 hr at room temperature followed by three washes with PBS to remove excess antigen. The detecting anti-CTX antibody specific to the JKLM region of the CTX molecule conjugated to horseradish peroxidase was added to each 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 µl/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 µl/well 1 N sulfuric acid. Absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Winooski, VT).

Scoring ELISA Data

To determine the borderline and positive threshold scores for ELISA, the mean optical density (OD) and the standard deviation of triplicate wells run with fish extract using the ELISA were obtained and compared with the six to eight replicate wells run on each plate using both the previously described nontoxic fish extract as well as various dilutions of the positive CTX extract obtained from the DOH Epidemiology Branch. Results were extrapolated to reflect the results obtained from multiple intra- and inter-assay runs using the nontoxic fish extract in which the average OD was found to be 0.100 ($n = 42$). A "positive" cut-off score was established for CTX by multiplying the mean OD of the nontoxic fish extract by 2, and samples were considered positive using the ELISA if the mean value scored above the positive threshold or 0.200 OD. Borderline or "$\pm$" values for CTX presence included values that fell within 0.020 OD of the established positive threshold or between 0.180 and 0.200 OD. Samples were considered "negative" using the ELISA if the mean value fell below the borderline threshold of 0.180 OD. Relative toxicity is expressed as the OD reading multiplied by 1,000.

NB Cell Bioassay for CTX Detection

The NB cell bioassay for the detection of CTX was modified after the method of Manger et al. (13). Mouse NB cells (neuro-2a, CCL-131, ATCC, Manassas, VA) were grown in Roswell Park Memorial Institute 1640 media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM glutamine, 1 mM sodium pyruvate, 50 µg/l streptomycin, and 50 units/ml penicillin. Cultures were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere. NB cells from stock culture were seeded in complete media in 96-well plates at a concentration of 1.5×10^5 cells/ml in 100 µl volumes per well and maintained at 24 hr at 37°C. After 24 hr, 10 µl each of 10 mM ouabain and 1 mM veratridine and 0.1 mg/ml of lipid extract from each fish sample were added to wells in triplicate. Control wells of ouabain/veratridine (OV) alone ($n = 8$) 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 activity, depicted by enhanced cytotoxicity in the presence of both CTX and OV. End-point assessment using (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium was carried out, and color end point was read at a wavelength of 490 nm using a microplate reader. Reagents were purchased from Sigma Chemical unless otherwise indicated.

Scoring NB Data

Using the NB assay, the cytotoxicity of each fish sample was measured as the percentage of surviving cells as indicated by OD readings where decreased OD values indicate increased cytotoxicity. Data were obtained for

analysis by comparing the average OD of wells containing cells, fish extract, and ouabain and veratridine with the average OD of control wells containing cells and ouabain and veratridine alone. Results were assessed by one-way analysis of variance followed by Tukey's post-test for multiple comparisons. P values below 0.05 were considered to be significant for CTX activity, and were scored as "positives" using the NB assay. Post-test critical values for significance that were above 3.50 as determined by GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA) but that fell below the established criteria for significance at $P<0.05$ were scored as "approaching significance," as the triplicate averages of these samples showed cell death when compared with the OV control, indicating some measure of toxicity within the sample. Values that scored "not significant" using these criteria were scored as "negative." Relative toxicity is expressed as the OD reading multiplied by 1,000.

Calculations and Statistical Analysis

Chi-square analysis was used to estimate the positive and negative percent agreements (analogous to sensitivity, defined here as the assay's ability to detect a low concentration of a given substance in a biological sample and, specificity, defined as the ability of an assay to exclusively identify a target substance or an organism rather than similar but different substances (20)) and associated confidence intervals of the ELISA when compared with the NB assay. Pearson's correlation and χ^2 analysis were used to compare the results of the ELISA and NB assays.

All statistical analyses were carried out using GraphPad Prism 5.0 Software. Results were regarded as significant at $P<0.05$ unless otherwise indicated.

RESULTS

ELISA Results

Analysis indicated that the ELISA had the capability of reliably detecting CTX-positive extract at concentrations of 0.016 mg/ml and higher. The dose response data for the CTX-positive extract (Fig. 2) were used in establishing the thresholds for CTX borderline and positive scores for evaluating the toxicity of individual fish samples.

When evaluated for CTX using the ELISA, 14.3% (25/175) of the roi samples yielded positive or borderline results (15/175, or 8.6% positive, and 10/175, or 5.7% borderline). Of the kahala tested for CTX with the ELISA, 4.3% (7/164) gave positive or borderline results (6/164, or 3.7% positive, and 1/164, or 0.6% borderline). The recorded OD values obtained for the borderline and positive scores using the ELISA and NB assays for roi and kahala are summarized in Tables 1 and 2.

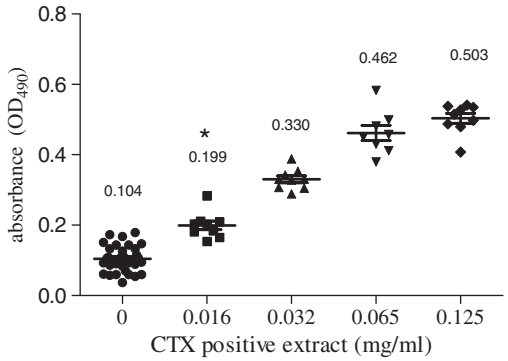

Fig. 2. ELISA results with average OD values from CTX-positive fish extract at various concentrations. ELISA, enzyme-linked immunoassay; OD, optical density; CTX, ciguatoxin.

NB Assay Results

Using the NB cell bioassay, 8% of the roi (14/175) tested significantly positive using strict statistical criteria when compared with the controls. An additional 4% (7/175) gave results indicative of cell death "approaching significance" when compared with the control and were classified as borderline. Thus, a total of 12% of the roi samples (21/175) gave results indicative of CTX presence using this method (Table 1). Using the NB cell bioassay, 2.4% (4/164) of the kahala tested significantly positive using strict statistical criteria when compared with the controls, and 1.2% (2/164) showed signs of cell death approaching significance using the NB cell bioassay, for a total of 3.6% (6/164) considered positive for CTX with this method (Table 2).

Comparison of ELISA and NB Assay Results

A total of 32 positive or borderline scores were obtained using the ELISA from all fish species ($n = 339$). Of those positive or borderline fish, 23 were scored as significantly positive or having cell death approaching significance using the NB assay. The remaining nine were scored as "not significant" using the NB assay, leading to a scoring of 2.7% implied false positives when using the ELISA. Additionally, four of the samples tested negative using the ELISA, but were scored as significantly positive or having cell death approaching significance using the NB assay, implying an overall score of 1.2% false negatives when using the ELISA.

The positive percent agreement of the ELISA as calculated using the NB and ELISA data was 85% (95% CI: 0.65–0.95), and the negative percent agreement was 96% (95% CI: 0.93–0.97). Correlation analysis comparing the OD results of the ELISA and the OD results of

TABLE 1. Results of ELISA and NB Assay Detecting Ciguatoxin in Roi Muscle Tissue

Sample ID	ELISA result (average OD ± SD) $n = 3$ Positive and borderline above 0.200 ± 0.020	ELISA score	NB control value (average OD ± SD) $n = 6$	NB result (average OD ± SD) $n = 3$	Statistical score (ANOVA)
Kona roi					
3	0.186 ± 0.025	Borderline	0.197 ± 0.015	0.170 ± 0.027	NS^
5	0.208 ± 0.031	Positive	0.197 ± 0.015	0.214 ± 0.011	NS
21	0.186 ± 0.007	Borderline	0.261 ± 0.017	0.126 ± 0.008	***
42	0.203 ± 0.016	Positive	0.261 ± 0.017	0.215 ± 0.019	NS^
61	0.206 ± 0.022	Positive	0.261 ± 0.017	0.188 ± 0.002	*
83	0.260 ± 0.011	Positive	0.261 ± 0.017	0.110 ± 0.002	***
93	0.185 ± 0.007	Borderline	0.261 ± 0.017	0.203 ± 0.021	NS^
103	0.184 ± 0.004	Borderline	0.183 ± 0.015	0.234 ± 0.028	NS
104	0.187 ± 0.014	Borderline	0.183 ± 0.015	0.198 ± 0.026	NS
107	0.224 ± 0.003	Positive	0.261 ± 0.017	0.181 ± 0.025	*
115	0.203 ± 0.010	Positive	0.235 ± 0.036	0.260 ± 0.005	NS
126	0.183 ± 0.007	Borderline	0.261 ± 0.017	0.118 ± 0.024	**
127	0.245 ± 0.015	Positive	0.261 ± 0.017	0.168 ± 0.003	*
138	0.226 ± 0.020	Positive	0.261 ± 0.017	0.185 ± 0.014	NS^
141	0.263 ± 0.019	Positive	0.261 ± 0.017	0.193 ± 0.028	NS^
143	0.263 ± 0.031	Positive	0.261 ± 0.017	0.215 ± 0.002	NS^
149	0.176 ± 0.004	Negative	0.261 ± 0.017	0.145 ± 0.008	***
172	0.203 ± 0.005	Positive	0.261 ± 0.017	0.126 ± 0.020	***
177	0.204 ± 0.032	Positive	0.261 ± 0.017	0.166 ± 0.022	**
182	0.243 ± 0.024	Positive	0.261 ± 0.017	0.142 ± 0.005	***
183	0.196 ± 0.030	Borderline	0.261 ± 0.017	0.159 ± 0.012	***
Oahu roi					
60	0.185 ± 0.044	Borderline	0.194 ± 0.026	0.214 ± 0.004	NS
54	0.104 ± 0.006	Negative	0.145 ± 0.028	0.102 ± 0.008	*
135	0.203 ± 0.018	Positive	0.182 ± 0.008	0.108 ± 0.024	*
139	0.207 ± 0.012	Positive	0.198 ± 0.022	0.164 ± 0.018	*
146	0.181 ± 0.029	Borderline	0.198 ± 0.022	0.168 ± 0.003	NS^
155	0.189 ± 0.017	Borderline	0.204 ± 0.045	0.219 ± 0.013	NS

Positive and borderline ELISA scores were obtained using a threshold value as calculated and described in the Materials and Methods section. NS: no significant change in cell death when compared with the control; NS^: cell death with respect to the control approaching levels of significance; and (*), (**), (***): significant increase in cell death when compared with the control ($0.01 < P < 0.05$, $0.001 < P < 0.01$, $P < 0.001$, respectively). Samples not included in this table tested negative using both methods ($n = 148$). ELISA, enzyme-linked immunoassay; NB, neuroblastoma; OD, optical density; ANOVA, analysis of variance.

the NB assay, depicted in Figure 3, showed negative correlation ($R = -0.39$) and statistical significance ($P < 0.001$). Chi-square analysis comparing the distribution of positive and negative results obtained with the ELISA and the NB assay did not show significant differences between the results ($P = 0.260$).

DISCUSSION

ELISA has become an indispensable tool for quantitative and semi-quantitative analysis for the detection of specific antigens in a variety of matrices. Several models for determining positive–negative thresholds using ELISA have been reported, including 2 and 3 times the mean of a nontoxic negative control and the mean of the nontoxic negative control plus two, three, and/or four standard deviations (21,22). Receiver–operator characteristic curves have also been used to give statistically optimal cut-offs and to assist in visualizing the tradeoffs between high sensitivity and specificity. However, the selection of actual cut-off determinations using immunological methods is generally mandated by practical realities rather than statistical optimization. In practice, cut-offs may need to be lowered or adjusted in order to not misclassify samples as false negatives. Although such an action may decrease the specificity of the test, thereby increasing the incidence of false-positive results, in situations where devastating economic or health implications are possible, this situation is preferred. It is recognized that the common use of such thresholds is arbitrary and generally should rely on a secondary assay to assist in determining the optimal threshold, which in this study was the implementation of the NB cell bioassay.

TABLE 2. Results of ELISA and NB Assay Detecting Ciguatoxin in Kahala Muscle Tissue

Sample ID	ELISA result (average OD ± SD) $n = 3$ Positive and borderline above 0.200 ± 0.020	ELISA score	NB control (average OD ± SD) $n = 6$	NB result (average OD ± SD) $n = 3$	Statistical score (ANOVA)
Kahala from MHI and NWHI					
MHI 8.3	0.189 ± 0.013	Borderline	0.173 ± 0.046	0.162 ± 0.008	NS
MHI 8.4	0.247 ± 0.022	Positive	0.173 ± 0.046	0.207 ± 0.049	NS
NWHI 3	0.205 ± 0.004	Positive	0.261 ± 0.017	0.138 ± 0.017	**
NWHI 5	0.208 ± 0.018	Positive	0.261 ± 0.017	0.181 ± 0.019	*
NWHI 6	0.229 ± 0.013	Positive	0.261 ± 0.017	0.175 ± 0.001	*
NWHI 7	0.271 ± 0.017	Positive	0.261 ± 0.017	0.164 ± 0.034	**
NWHI 11	0.200 ± 0.005	Positive	0.248 ± 0.047	0.235 ± 0.012	NS
NWHI L22	0.058 ± 0.011	Negative	0.249 ± 0.048	0.178 ± 0.008	NS^
NWHI KP 14	0.114 ± 0.021	Negative	0.156 ± 0.036	0.065 ± 0.008	NS^

Positive and borderline ELISA scores were obtained using a threshold value as calculated and described in the Materials and Methods section. NS: no significant change in cell death when compared with the control; NS^: cell death with respect to the control approaching levels of significance; and (*), (**), (***): significant increase in cell death when compared with the control ($0.01 < P < 0.05$, $0.001 < P < 0.01$, $P < 0.001$, respectively). Samples not included in this table tested negative using both methods ($n = 155$). ELISA, enzyme-linked immunoassay; NB, neuroblastoma; MHI, main Hawaiian Islands; NWHI, northwestern Hawaiian Islands; OD, optical density; ANOVA, analysis of variance.

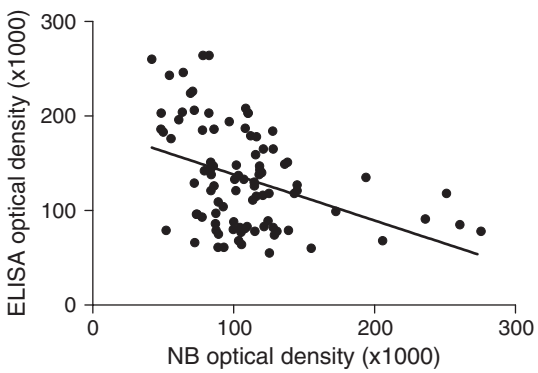

Fig. 3. Correlation analysis comparing ELISA and NB optical density (OD) results where increasing OD values indicate toxicity using the ELISA and decreasing OD values indicate toxicity using the NB assay ($R = -0.39$, $P < 0.001$). ELISA, enzyme-linked immunoassay; NB, neuroblastoma.

The positive threshold for the ELISA in this study was established to be 2 times the mean OD of the nontoxic fish extract based in part on multiple assay runs using both the nontoxic fish extract and a previously characterized partially purified positive CTX extract. The comparison of ELISA results for partially purified CTX-positive extract and nontoxic fish extract as negative control showed that the method was able to reliably detect CTX at 0.016 mg/ml (Fig. 2), which correlates to approximately 5 pg/ml of equivalent CTX per mg of extract. This concentration of toxin is considered subclinical and ingestion at this level would not likely result in mammalian illness. At 0.016 mg/ml, the CTX-positive extract gives an average OD of 0.200 ($n = 15$), which is approximately double the average OD reading obtained when running the nontoxic fish extract with the ELISA (0.100, $n = 42$). Although the establishment of cut-offs in ELISA is arbitrary to some degree as indicated, these results were useful in establishing a cut-off for positivity.

One of the pitfalls of ELISA is the sequential accumulation of small random errors such as small differences in incubation times, calculation of standards and samples, and slight differences in reagents through the course of the experiment, which culminates in color development and OD measurements that are consequences of all the previous steps. Although experiments were closely controlled to maintain uniformity and minimize such errors, data from each plate were extrapolated to reflect the 0.100 OD negative control reading and the cut-off for CTX positivity was named as 2 times the negative control or 0.200 OD in this study. The lack of availability of reliable purified CTX standards to further validate the ELISA may affect the interpretation of the results with respect to determination of the true levels of CTX present in the partially purified CTX-positive fish extract and crude fish samples, and leaves the door open for further improvement of the method in the future.

Owing to the preliminary and novel status of the ELISA, the NB assay was performed as a secondary screen. As a potent sodium channel activator, CTX, if present in crude fish extract even in picogram quantities, in combination with the chemical agonists ouabain and

veratridine (OV), will lead to quantifiable NB cell death (13). The NB assay lacks specificity in that the results represent a functional mechanism rather than structural recognition of a particular antigen or molecule, but nonetheless it is considered a reliable method for the detection of CTX at present as gauged by its applied use in previous studies (13,23–27).

According to the US Food and Drug Administration, when reference standards are not available, estimates of sensitivity and specificity can be made using a secondary method and are reported as positive and negative percent agreements (28). Results of the ELISA and NB were highly correlated ($P<0.001$, Fig. 3), and comparison of the results indicated that the ELISA has a positive percent agreement of 85% and a negative percent agreement of 96%, which can be considered reasonable for a biological assay of this type. In addition, the false-positive (2.7%) and false-negative (1.2%) rates were low. The occurrence of false positives likely results from the somewhat arbitrary determination of the positive threshold; however, it is preferable to err on the side of caution and report false positives rather than false negatives when detecting a potentially harmful substance such as CTX. It is important to note that using the NB assay results to confirm or refute the ELISA results assumes that the NB assay results are completely accurate, which in practice is open to discussion owing to the nonspecific nature of the assay and the potential for error during administration. A tertiary evaluation of a subset of the positive and negative samples using a chemical evaluation method for CTX such as liquid chromatography-mass spectrometry (3) could be used in the future to further narrow down estimates for precision and accuracy, and to provide improved sensitivity and specificity values by refining the cut-off value.

In conclusion, the newly developed ELISA has reasonable specificity and sensitivity for an immunological screening assay, and the comparison with the accepted NB assay indicated that it is a valid new tool for screening fish for CTX. The first application of the new method on samples of the fish species *S. rivoliana*, *S. dumerili*, and *C. argus* in this study demonstrates its potential in the large-scale screening of fish populations for CTX in the future.

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REFERENCES

1. Lehane L, Lewis RJ. Ciguatera: Recent advances but the risk remains. Int J Food Microbiol 2000;61:91–125.
2. Ting JYS, Brown AFT. Ciguatera poisoning: A global issue with common management problems. Eur J Emerg Med 2001;8: 295–300.
3. 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.
4. Sasaki DS. 2001. Ciguatera fish poisoning: A five-year review. Communicable Disease Report, Hawaii Department of Health, 4–6 September/October.
5. Anderson BS, Sims JK, Wiebenga NH, Sugi M. The epidemiology of ciguatera fish poisoning in Hawaii, 1975–1981. Hawaii Med J 1983;42:326–334.
6. Yasumoto T, Oshima Y, Murakami Y, Nakajima I, Bagnis R, Fukuyo Y. Toxicity of benthic dinoflagellates. Bull Jpn Soc Sci Fisheries 1980; 46:327–331.
7. Mines D, Stahmer S, Shephard SM. Poisonings food, fish, shellfish. Emerg Med Clin North Am 1997;15:157–177.
8. Swift AEB, Swift TR. Ciguatera. Clin Toxicol 1993;31:1–29.
9. Randall JE. Introductions of marine fishes to the Hawaiian Islands. Bull Mar Sci 1987;41:490–502.
10. Randall JE, Earle JL, Pyle RL, Parrish JD, Hayes T. Annotated checklist of the fishes of Midway Atoll, Northwestern Hawaiian Islands. Pac Sci 1993;47:356–400.
11. Bruno PP, Effler PV. 2001. Summary of reported cases of notifiable diseases, Hawaii, 1995–2000. Communicable Disease Report: 8.
12. Earle JL. Have we created a monster? Hawaii Fishing News 2005;31:14.
13. 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.
14. Hokama Y, Takenaka WE, Nishimura KL, Ebesu JSM, Bourke R, Sullivan PK. A simple membrane immunobead assay for detecting ciguatoxin and related polyethers from human ciguatera intoxication and natural reef fishes. J AOAC Int 1998;81:727–735.
15. 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.
16. Tamaru CS, Anderson B, Hokama Y, Vincent D. 2005. Evaluating the risk of ciguatera fish poisoning from consumption of reef fish grown at marine aquaculture facilities in Hawaii. World Aquaculture Society Conference, Bali, Indonesia, 9–13 May.
17. Campora CE, Hokama Y, Yabusaki K, Isobe M. Development of an enzyme-linked immunosorbent assay for the detection of ciguatoxin in fish tissue using chicken immunoglobulin Y. J Clinl Lab Anal 2008;22:239–245.
18. Kimura LH, Abad MA, Hokama Y. Evaluation of the radioimmunoassay (RIA) for detection of ciguatoxin (CTX) in fish tissues. Fish Biol 1982;21:671–679.

19. 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.
20. Saah AJ, Hoover DR. Sensitivity and specificity reconsidered: The meaning of these terms in analytical and diagnostic settings. Ann Intern Med 1997;126:91–94.
21. Sutula CL, Gillet JM, Morrissey SM, Ransdel DC. Interpreting ELISA data and establishing the positive–negative threshold. Plant Dis 1986;70:722–726.
22. Fenlon JS, Sopp PI. Some statistical considerations in the determination of thresholds in ELISA. Ann Appl Biol 1991; 119:177–189.
23. LePage KT, Dickey RW, Gerwick WH, Jester EL, Murray TF. On the use of neuro-2a neuroblastoma cells versus intact neurons in primary culture for neurotoxicity studies. Crit Rev Neurobiol 2005;17:27–50.
24. Garrec RB, Benoit E, Sauviat MP, Lewis RJ, Molgo J, Laurent D. Ability of some plant extracts, traditionally used to treat ciguatera fish poisoning, to prevent the in vitro neurotoxicity produced by sodium channel activators. Toxicon 2005;46:625–634.
25. 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.
26. Dechraoui MY, Naar J, Pauillac S, LeGrand AM. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. Toxicon 1999;37:125–143.
27. Manger RL, Leja S, Lee SY, Hungerford JM, Wekell MM. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: Semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal Biochem 1993;214:190–194.
28. FDA. Statistical Guidance on Reporting Results from Studies Evaluating Diagnostics Tests; Draft Guidance for Industry and FDA Reviewers. FDA Web site, accessed on 14 February 2008, www.fda.gov/cdrh/osb/guidance/1428.html, 2007.