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Competitive ELISA: An Accurate, Quick and Effective Tool to Monitor Brevetoxins in Environmental and Biological Sample

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Abstract

A competitive Enzyme-Linked Immuno-Sorbent Assay (competitive ELISA) has been developed for analyzing brevetoxins (PbTxs). Antibodies to brevetoxins were used in combination with a multi-step signal amplification procedure for the detection of toxins. This procedure minimizes non-specific signals and background noise often observed in complex matrices. Therefore, analysis can be performed with various samples (seawater, air filter, mammalian body fluids, shellfish, etc.) without the need for extensive extraction and/or purification steps. Brevetoxin analysis in liquid samples like seawater, urine and serum can be performed without pretreatment, dilution or purification. The limit of quantification of PbTxs is 2 ng mL^{-1} in any of the liquid sample matrices tested. For shellfish monitoring, analyses are performed after homogenization of shellfish meat (5 g) with brevetoxin-ELISA buffer (200 mL) and can be performed on tissue from a single mollusk as well as on a pool of shellfish meat. Comparative quantification of PbTxs achieved in buffer, seawater, mammalian body fluid and shellfish homogenate spiked with equal amounts of toxin (10 ng mL^{-1} sample) varied by no more than 5%. These data suggest that the matrix composition of the sample does not affect the performance of the assay. Because this assay is not affected by matrix composition and can be performed in shellfish homogenate, this procedure can be used to prevent or diagnose human exposure to PbTxs and has the potential to replace the currently used mouse bioassay for monitoring PbTxs in shellfish.

Introduction

Almost every year, and sometimes several times in the same year, blooms of the dinoflagellate *Karenia brevis* are observed in the Gulf of Mexico. These blooms, also called red tides, usually affect the west coast of Florida, but on several occasions have had an impact on every state that borders the Gulf of Mexico. Red tides appear to have had a long history in Florida, with the earliest recorded event dating to the 1800s. Red tides almost always result in fish kills and temporary closures of local shellfish harvesting areas. Local tourism activities such as boating, recreational fishing and beach-related activities are also negatively affected. Red tides are a threat to both human and environmental health. *K. brevis* produces brevetoxins, potent neurotoxins that are found in the organisms at a concentration of approximately 10 pg $cell^{-1}$ as well as in the seawater supporting blooms. In addition to

Steidinger, K. A., J. H. Landsberg, C. R. Tomas, and G. A. Vargo (Eds.). 2004. Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO.

the health effects associated with neurotoxic shellfish poisoning, there have been multiple anecdotal reports of respiratory irritation and possibly immunologic effects associated with the inhalation of aerosolized seawater during Florida red tides. Recent die-offs of the endangered Florida manatee have also been associated with brevetoxins. Research in sheep and other laboratory animals has confirmed the ability of aerosolized red tide toxins to cause reversible bronchospasm. Brevetoxins induce toxicity at very low concentrations. Therefore, effective monitoring requires an analytical procedure having sufficient sensitivity and specificity to detect toxin at sub-symptomatic levels. Since the matrices in which the toxins are found (algae, shellfish, body fluid and seawater) are diverse and complex, current methods of quantitative analysis are laborious and imprecise, requiring many steps of extraction and purification. The difficulties in quantifying brevetoxins in biological samples have led us to pursue an alternate analytical approach. This study reviews the use of the sensitive and accurate ELISA method developed in recent research on shellfish management and monitoring, and human and animal exposure to aerosolized brevetoxins (Naar *et al.*, 2002).

Materials and Methods

Competitive ELISA

Development of this method is fully described in Naar *et al.* (2002).

A: Liquid samples (seawater, urine, serum, etc.)—Samples were serially diluted (dilution factor = 2) with buffer into the sensitized ELISA plate. Anti-brevetoxin antibodies were added into all the wells and incubated for 1 hour. After incubation, the plate is washed, and the revelation of immobilized antibodies is obtained by successive addition of the secondary antibody, the horseradish peroxidase conjugate, and the enzyme substrate. The reaction is terminated by addition of sulfuric acid. Absorbance is recorded at 492 nm to quantify the amount of brevetoxin present in the sample.

B: Shellfish samples—Shellfish meat was homogenized in buffer using a commercial blender. The homogenate can be analyzed directly using the protocol describe above. Alternatively, brevetoxins can be extracted from shellfish with organic solvents (ethyl ether or acetone). After extraction, the residue is dried and dissolved in buffer for analyses as performed above.

Results and Discussion

Exposure to aerosolized brevetoxins

The Gulf Coast is a region highly dependent on tourism and other coastal industries. Faced with intermittent aerosol exposures resulting in possible acute and chronic respiratory effects, a new public health and epidemiologic investigation has been instituted in Florida. To investigate the human health effects of environmental exposure to red tide toxins, an interdisciplinary team of scientists has been formed (Fleming *et al.*, 2003). When a red tide moves near shore where people might be exposed, this team rapidly assembles at the site to collect environmental samples and epidemiologic data.

With the development of the competitive ELISA methodology for brevetoxin analysis, levels of contamination by brevetoxins in water and in sea sprays (Chung *et al.*, 2003) were precisely measured on the day samples were collected. During moderate and high exposure periods in Jacksonville, FL, brevetoxin concentrations of 36 to 80 ng/m^3 were measured in the air (Pierce *et al.*, 2003). An average adult breathes in 25 L min^{-1} of air during light exercise. People visiting the beaches during these periods were inhaling up to 54–120 ng of brevetoxin hr^{-1}, or an inhaled dose of 0.77–1.71 ng kg^{-1} hr^{-1} (Backer *et al.*, 2003). During the same period, using the ELISA methodology, analysis of air filters from a personal air sampler revealed a direct correlation between presence on the beach and exposure to brevetoxins (unpublished data).

During the fall of 2001, an extensive Florida red tide was studied off the coast of Sarasota, Florida. The data obtained included *K. brevis* counts in the water, the amount of toxin in the water, toxin in aerosols transported on shore, subsequent exposure of humans through respiration, and both throat swab and epidemiologic data on occupationally exposed individuals (lifeguards). These data were supported by meteorological measurements. ELISA was used to quantify total toxin on site. ELISA and LC-coupled mass spectrometry were used subsequently in the lab (Baden *et al.*, 2003). During the 5-day study, toxin concentrations in the water were measured at six different locations and ranged from 20 ng mL^{-1} to 400 ng mL^{-1}. *K. brevis* cell counts ranged from 1,000 to 15 million cells L^{-1}. The amount of toxin on impact air sampler filters was 80–467 ng cm^{-2}. Symptoms of sneezing, eye irritation, and coughing were experienced by the lifeguard subjects and scientists. Toxin levels and symptoms were inversely correlated with distance from the shoreline (Baden *et al.*, 2003).

During calm weather, offshore seawater samples show a strong correlation between cell concentration and toxin amount in the water. However, the cell counts from inshore water samples (at the beach) are not a good predictor of the toxin concentration (unpublished data). It is suspected that wind and wave action, combined with low water depth, induce cell lysis and subsequent accumulation of brevetoxins in the water. Brevetoxin concentrations can be high even as the cell densities remain low. Thus, we believe that measuring the cell abundance to monitor water quality and predict respiratory irritation and possible deleterious health consequences at the beach should be replaced by toxin analysis.

Management and Monitoring

Prevention of Neurotoxic Shellfish Poisoning in the United States relies upon environmental monitoring for *K. brevis* and timely closure of affected shellfish resources. The reopening of resources is contingent upon results from mouse bioassay of extracts from exposed shellfish. Recent research has shown that brevetoxins are rapidly accumulated and metabolized by shellfish and that the mouse bioassay method does not account for potential toxicity of the metabolites. With the goal of replacing the mouse bioassay, a multi-laboratory comparative study was undertaken to test the accuracy and precision of four alternative methods for the determination of brevetoxins in shellfish. These include the N2a neuroblastoma cytotoxicity assay, two variations of the sodium channel receptor binding assay, the competitive ELISA, and LC/MS (Dickey *et al.*, 2003). Results of this study indicate that the ELISA methodology

is the best candidate to replace the mouse bioassay. Because of its sensitivity, ELISA analysis can be performed on a single or even part of a single bivalve mollusk. Individual analyses of shellfish harvested at the same time from the same bed have shown a low variability in toxin concentration (Weidner *et al.*, 2003), indicating monitoring of shellfish by ELISA can be very precise while reducing the time and cost of analysis.However, depending on the protocol used (homogenization or organic solvent extraction), parent brevetoxins and brevetoxin metabolites can be measured together or individually in shellfish (Naar *et al.*, 2003). We believe that the regulatory agencies should seriously consider evaluating the different types of toxins that need to be monitored to prevent human intoxication, whether they are parent toxins, toxin metabolites or both.

Since the discovery of other brevetoxin-producing algae in US coastal waters (Bourdelais *et al.*, 2002), brevetoxin production by algae from the genera *Chattonella* and *Fibrocapsa* has been described (Bridgers *et al.*, 2003). Again, the ELISA methodology was used to rapidly and precisely measure brevetoxin production by theses organisms.

The competitive ELISA assay is reliable, inexpensive, quantitative, and not limited to a single application (*i.e.*, no matrix effects are observed). Since its development in 2002, this assay has already been used by several laboratories and regulatory agencies. Current applications include diagnosis of exposure in birds, marine mammals and humans as well as monitoring of shellfish, seawater and sea aerosols.

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