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RESEARCH ARTICLE

Screening of Ciguatoxins in the Philippines by Animal Assay: Symptoms, Levels, and Distribution in Fish Tissue

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ABSTRACT

The Philippines is an archipelagic country that belongs to the biologically diverse Pacific Coral Triangle, rich in marine resources, including corals, reef fishes, and algae. This explains the continuous sustenance of the Filipinos on fish as a major protein source. Despite their contribution to human consumption, some commercially important coral reef fishes are a threat to food safety, compromising public health. Currently, ciguatera fish poisoning (CFP) has been focused on by scientists since it is the most frequently reported seafood-toxin illness in the world acquired from contaminated coral reef fishes. The present study investigates the contamination of reef fishes in the West Philippine and Sulu Seas using animal assay. Ciguatoxins (CTX) are present in commercially important reef fishes such as barracuda (*Sphyraena barracuda*), parrotfish (*Scarus quoyi*), rabbitfish (*Siganus guttatus*), grouper (*Plectropomus leopardus*), moray eel (*Gymnothorax melanospilos*), and snapper (*Lutjanus campechanus*). *Scarus quoyi* had the highest toxicity of 0.65 ± 0.55 ppb and 0.48 ± 0.36 ppb found in flesh and viscera, respectively. Although higher toxicities were observed from fish viscera, toxicities between fish parts did not vary greatly ($p > 0.05$). Positive samples exceeded the 0.01 ppb guideline established by the US Food and Drug Administration and the Philippines' regulatory limit set by the Bureau of Fisheries and Aquatic Resources. Symptoms of mice showing the presence of Pacific CTX-1 were noted. Since mouse bioassay was used in screening reef fishes that pose non-specificity and insensitivity problems, the researchers suggest that analytical methods must be used in characterizing and quantifying these types of toxins. Establishing the methodologies in detecting CTX would greatly help monitor and manage CFP in commercially identified reef fishes in the country.

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1. INTRODUCTION

The potential world catch from coral reefs is reported at 5.54 million metric tonnes per year (Lecaillon et al. 2000). This is equivalent to 6.60% of the current world capture production that is 81.65 million metric tonnes (FAO 2018). In the Philippines, productions of reef fishes like groupers and siganids in 2018 have been reported at 17,671.43 metric tonnes and 23,267.63 metric tonnes, valued at 4.61 and 2.28 billion pesos, respectively (PSA 2019). In addition, 14,364.65 metric tonnes of snapper were produced, valued at 1.94 billion pesos. Despite their contribution to human consumption as a protein source, some commercially important major fish groups from coral reefs are threats to food safety, compromising public health. Biotoxins, including ciguatoxins, are detected from these reef fishes may cause poisoning to people (Higman et al. 2014).

Ciguatera fish poisoning (CFP) has been focused on by scientists since it is the most frequently reported seafood-toxin illness in the world acquired from contaminated coral reef fishes (Friedman et al. 2008). Ingestion of toxic reef fish causes gastrointestinal, neurological, and cardiovascular symptoms that can last for several days or months (Lewis 2001; FAO 2004; CDC 2006; EFSA 2010). It was estimated that 10,0000–50,000 persons per year are affected by this type of poisoning (Friedman et al. 2008; Soliño and Costa 2020). While many thousands

of people are affected annually, the poisoning is rarely fatal (0.1%), although it is thought that the proportion of CFP cases reported is low (CDC 2006; Tubaro et al. 2012).

In a study conducted by Dionisio (2018), 17 related CFP outbreaks affecting 146 individuals occurred from 1988 to 2010 in the Philippines. High-risk areas were found to be in the islands of Palawan and Sibuyan in Luzon, the island of Panay in the Visayas, and Basilan in Mindanao. The most recent case was reported in Zamboanga City in Mindanao last 2014, wherein 14 patients experienced shortness of breath, dizziness, and vomiting after eating barracuda fishes (Azanza et al. 2019). Recently, a study was conducted by Montojo et al. (2020) documenting the presence of ciguatera and ciguatera-like toxins in reef fishes in the Visayan and Sibuyan Seas. According to the study, toxicity was site-specific, i.e., geographical conditions greatly affect the presence of toxic organisms such as *Gambierdiscus, Ostreopsis,* and *Prorocentrum* species, contaminating the reef fishes. Significant differences were noted between seasons. Furthermore, fish weight was not a good predictor of contaminated reef fishes. They documented the presence of *Ostreopsis* spp. in all sampling sites, which can possibly contaminate the reef fishes. Lastly, they stated the correlation of fish toxicity to the occurrence of toxic benthic dinoflagellates.

Since CTX is colorless, odorless, and tasteless, detection remains a challenging task for laboratories and regulatory bodies to identify contaminated fishes in the market (Wong et al. 2005). Traditional mouse bioassay is a widely used method in detecting this type of toxin, but with the advancement of technology, analytical and functional assays can also be used. The problem with analytical and functional assays is the unavailability of reference standards making these methods not feasible (Bottein Dechraoui et al. 2005). Hence, no standardized method has been established to date (Suzuki et al. 2017). In the absence of reliable, robust, and simple assays for rapid screening of potential ciguateric fish, traditional mouse bioassay (MBA) is still the mainstay for toxicity confirmation in fish in the markets and fish remnants from CFP patients (Wong et al. 2014).

Ensuring seafood safety is our primary concern. The present study investigates the contamination of reef fishes in the West Philippine and Sulu Seas. The objective was to determine levels of toxicity and distribution of ciguatoxins in fish tissue then compare these levels with the existing regulatory limits such as the U.S. Food and Drug Administration and the Philippines' Bureau of Fisheries and Aquatic Resources (BFAR) Fisheries Administrative Order (FAO) 210 series of 2001. Noted symptoms in mice for the detection of CTXs were also documented. The presence of such toxins in coral reef fishes has socio-economic impacts that significantly affect the fish trade industry and public health (Chan 2016).

2. MATERIALS AND METHODS

2.1 Study Area

The study area is focused on the West Philippine and Sulu Seas (Figure 1), specifically in the municipalities of Cuyo (N 10° 49.906' E 121° 0.204') and Quezon (N 11° 22.225' E 120° 51.906'), Palawan. These areas were chosen since most cases of CFP were reported from Panay Island and Cuyo Pass.

2.2 Collection of reef fish samples

Coral reef fish samples were collected in fish landing sites of Cuyo and Quezon, Palawan. Specifically, commercially important reef fishes were collected belonging to six genera such as barracuda (*Sphyraena barracuda*), parrotfish (*Scarus quoyi*), rabbitfish (*Siganus guttatus*), grouper (*Plectropomus leopardus*), moray eel (*Gymnothorax melanospilos*), and snapper (*Lutjanus campechanus*) from July 2012 to November 2013. Collection of samples were done in wet (November to April) and dry (May to October) seasons of Palawan. The fishes were photographed and measured for standard length and weight, dissected into flesh and viscera, and frozen at -20°C until use. All fish samples were identified using the book of Gonzales (2013).

2.3 Toxicity in reef fishes

Toxin extraction and mouse bioassay (MBA) followed the procedure described by Lewis (1995). One hundred to 1000 g of fish flesh and viscera were kept for assessment of toxicity. A series of solvent extraction was carried out using a rotary evaporator. Fish tissues and viscera were cooked for at least 15 minutes in a 70°C water bath. For every 100 g of sample, 300 mL of acetone were added twice for homogenization and initial extraction. This was followed by extraction using 50 mL of 90% methanol and 50 mL hexane. Then final extraction was carried out using ethanol-water (1:3) mixture and 50 mL diethyl ether. Chloroform-methanol (97:3) was used to dissolve the residues from the previous extraction. The solvent was evaporated using a rotary evaporator.

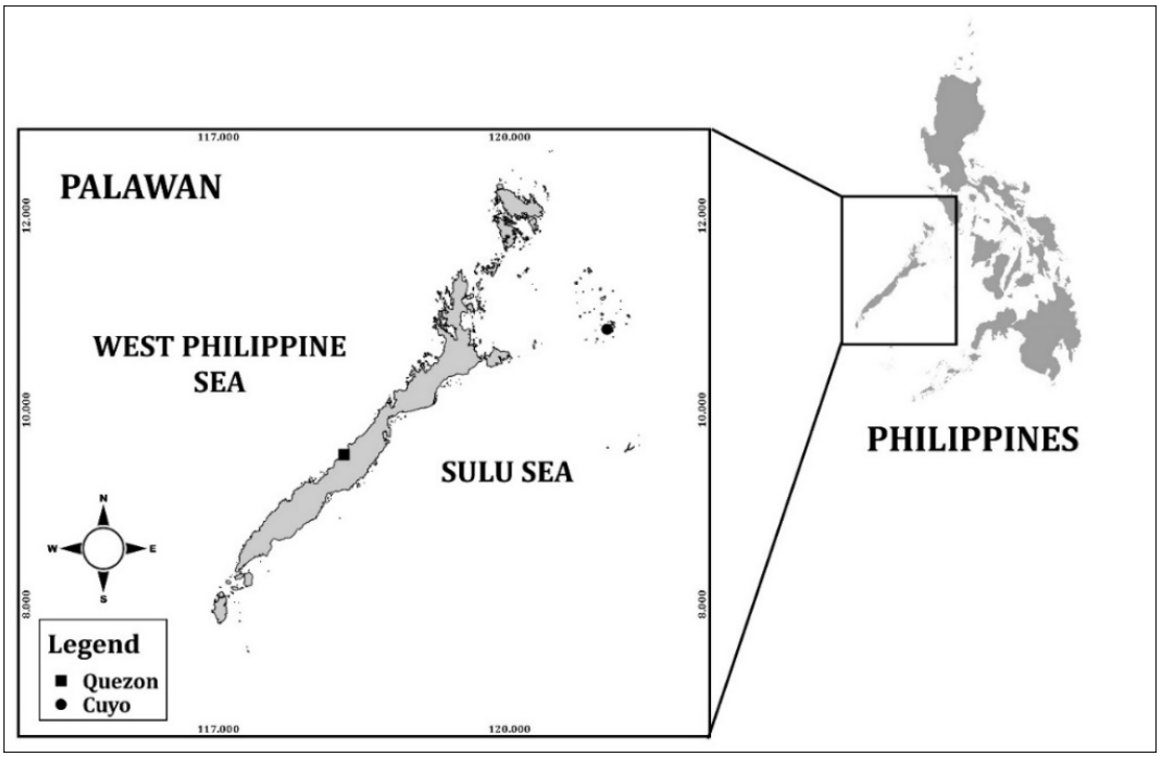

Figure 1. Map showing the sampling area

Lastly, the final residue was dissolved using a required amount of 5% Tween 60 in 0.9% saline solution to come up with a 40 g/mL concentration. Toxin extract was used for mouse bioassay analysis.

International Cancer Research (ICR) strain male mice obtained from the Food and Drug Administration – Department of Health of the Philippines weighing 17–20 g were used for mouse bioassay analysis. Mice were acclimatized in the laboratory for five days with a 12/12 hour light/dark cycle then provided with food and water *ad libitum*. Three trials per sample were done to confirm the presence of CTXs. An intraperitoneal (i.p.) injection was performed using 0.5 mL of the toxin extract. Moreover, negative controls were also set up, administering 0.5 mL of 5% Tween 60 in 0.9% saline solution in mice. Mice were closely monitored for the next two hours then intermittently over at least a 24-hour period. Samples were judged toxic if two to three mice died in the experiment. Toxicity was computed using the formula log MU = 2.3 log $(1+T^{-1})$ wherein 'T' should be in hours.

Same with the study conducted by Montojo et al. (2020), standards for ciguatoxins are still not available in the market; thus, judging the sample as positive will be based on the symptoms stated by FAO (2004) and EFSA (2010) for Pacific CTX-1 such as piloerection, diarrhea, lachrymation, hypersalivation, dyspnea, wobbly upright gait, and gasping.

2.4 Statistical analysis

Data analyses were completed using functions incorporated in the Statistical Package for the Social Sciences (SPSS) version 21. Significant differences were determined using Student's t-test in comparing toxicities between flesh and viscera and the occurrence of toxicity between the two areas.

3. RESULTS

3.1 Levels and distribution of CTX in fish tissues

Of the total 103 reef fish samples collected, 46 reef fishes or 44.66% tested positive with CTX. It was noted that contaminated samples had toxicities ranging from 0.26 to 1.73 ppb. Most samples tested positive for only one of its parts, i.e., flesh or viscera. But three specimens tested positive for both of its parts belonging to two *S. quoyi* and one *S. guttatus* thus having a total of 49 toxic specimens. Only four or 3.88% of the total samples analyzed tested positive

from fish flesh. *S. quoyi* had the highest toxicity of 0.65 ± 0.55 ppb followed by *L. campechanus* and *S. guttatus*. Additionally, no fish flesh tested positive from *G. melanospilos*, *P. leopardus*, and *S. barracuda*. Comparatively, all species of reef fish samples tested positive from fish viscera. *S. quoyi* had the highest toxicity of 0.48 ± 0.36 ppb among reef fishes. This was then followed by *S. guttatus, S. barracuda, L. campechanus, P. leopardus*, and *G. melanospilos.*

Ciguatoxins were detected in both areas, the West Philippine Sea and the Sulu Sea. As observed, higher toxicities were detected from the Sulu Sea (Table 1). *S. quoyi* had the highest toxicity of 1.04 ppb and 0.54 ppb in flesh and viscera, respectively. It was noted that no positive samples were detected from *G. melanospilos* and *S. barracuda*. Compared with the West Philippine Sea, collected samples from the Sulu Sea such as *S. guttatus* had the highest toxicity of 0.32 ppb in fish viscera. There were no positive samples from *L. campechanus.*

Table 1. Toxicity and distribution of CTX in fish tissues

Species	West Philippine Sea			Sulu Sea		
	Toxicity (ppb)					
	Examined (n)	Flesh	Viscera	Examined (n)	Flesh	Viscera
Gymnothorax melanospilos	2	0	0.26	4	0	0
Lutjanus campechanus	4	0	0	8	0.50	0.29
Plectropomus leopardus	11	0	0.27 ± 0.02	15	0	0.27
Scarus quoyi	13	0.26	0.29 ± 0.03	22	1.04	0.54 ± 0.42
Siganus guttatus	10	0	0.32 ± 0.06	11	0.28	0.47 ± 0.19
Sphyraena barracuda	3	0	0.29 ± 0.02	0	0	0
Total	**43**			**60**		

3.2 Confirmation of CTX through symptoms

As observed from mouse bioassay, affected mice exhibited typical signs of ciguatera, including body weakness or reduced locomotor activity, which was experienced by 73.47% of mice (Table 2). This was then followed by a wobbly upright gait, gasping or dyspnea, diarrhea, piloerection, hypersalivation, and lachrymation. Symptom such as hypothermia was not determined due to the lack of instrument needed. However, piloerection, a response of mice when subjected to low temperature as it would trap air and retain heat, was observed, suggesting hypothermia in mice. It was also noted that there was a decrease in the body weight of affected mice even though food and water were given *ad libitum*. Contrariwise, negative specimens showed body weakness and wobbly upright gait, but the mice survived for the next five days after intraperitoneal (i.p.) injection.

Table 2. Noted behavior in mice after intraperitoneal (i.p.) injection1

Mouse Symptoms	Percentage*
Hypothermia	Not determined
Piloerection	26.53
Diarrhea	34.69
Lachrymation	18.37
Hypersalivation	12.24
Gasping / dyspnoea	38.78
Wobbly upright gait	46.94
Body weakness	73.47

*Percentage is equal to the number of symptoms occurrence over the total number of positive samples

4. DISCUSSION

4.1. Levels and distribution of CTX

Historically, detection of ciguatoxins in the Philippines was carried out using Cigua-Check® test kits by the Bureau of Fisheries and Aquatic Resources (Montojo et al. 2008; Mendoza et al. 2013). However, this test kit may pose a problem due to ambiguity of test results (Bienfang et al. 2011). Thus, traditional mouse bioassay was used in this study.

The US Food and Drug Administration (FDA) currently proposed a guideline of 0.01 ppb to ciguatoxins. In the Philippines, a regulatory limit was set by BFAR FAO 210 s. 2001 wherein ciguatoxins should be negative (BFAR 2001). Observed levels of CTX from reef fishes in this study exceeded the proposed guideline of the two countries that was remarkable and concern of public health. Thus, management and monitoring are necessary to ensure seafood safety to the consumers. Many countries employed different strategies in combatting this kind of poisoning. European Union (EU) Regulation no. 854/2004 states that checks should be implemented to ensure that fishery products that may contain toxins, including CTX, are not placed on the market (Campbell et al. 2011; Hossen et al. 2015; EU 2004). Furthermore, US, Japan, Australia, and New Zealand practical measures are required for minimizing the risk of fish products containing CTX, including controls on the species of fish or areas of harvest. For French overseas territories, a list of fish species potentially contaminated with CTX is available which are not allowed to be marketed (Higman et al. 2014).

Contrariwise to the previously described study of Montojo et al. (2020), the proportion of toxic reef fishes in this study was considerably higher at 44.66% due to the fish parts analyzed. It should be noted that both fish flesh and viscera were analyzed; thus, a remarkable proportion of toxic reef fishes was notable. As observed, toxic fish flesh comprises only 3.88% of the total samples analyzed. This was close to the proportion of toxic fish flesh observed in the previous study of Montojo et al. (2020) at 4.46%; hence it was evident that a small proportion of toxicities can be observed from fish flesh. The minor discrepancies in the data may be attributed to the different extraction procedures used in the studies.

Overall, *S. quoyi* has the highest toxicity among reef fishes collected, followed by *S. guttatus, L. campechanus,* and other predatory reef fishes. Looking into the feeding habit of these fishes, it follows that herbivorous species are relatively more toxic than carnivorous species. This is inconsistent with the existing belief that carnivorous species are more harmful than herbivorous species (Caillaud et al. 2010).

Various marine toxins originate from microorganisms native to the aquatic environment. These molecules eventually find their way into the human gastrointestinal tract through concentrating and bioaccumulating reef fishes, mollusks, and crustaceans. To name a few, these are brevetoxins, palytoxins, maitotoxins, and tetrodotoxins. Some are lipophilic compounds such as brevetoxins, ciguatoxins, and palytoxins. This explains the close similarity between brevetoxins and ciguatoxins since they also both belong to a large family of stable lipid-soluble cyclic polyether compounds. Being lipophilic, these toxins and their more polar metabolites are concentrated primarily in the viscera, liver and gonads resulting in toxin accumulation up the marine food chain in more than 400 species of fish (Lehane and Lewis 2000) and up to the larger predatory fish which also remain unharmed.

4.2. Toxin distribution

In this study, fish toxicity in the flesh ranges from 0.26 to 1.04 ppb compared to toxicity in the viscera, ranging from 0.26 to 1.73 ppb. Although higher toxicities were observed from fish viscera, toxicities between fish parts were not that significant ($p > 0.05$). Results showed agreement from previous studies stating that CTX concentration was usually higher in fish viscera (particularly in the liver) than in fish flesh (Hossen et al. 2015; Soliño and Costa 2020). This explains the small likelihood of ciguatera related fatalities in people since fish flesh which was primarily consumed had smaller toxicity occurrences than fish viscera (Lehane and Lewis 2000). CTXs are liposoluble compounds that are not easily excreted by reef fishes, thus storing this type of toxin in their bodies for longer periods (Higman et al. 2014). Since the liver's primary function is biotransforming liposoluble xenobiotic substances into more water-soluble metabolites prior to excretion (Morey et al. 2008), it explains the observed higher concentrations of toxicity in fish viscera than that of the flesh.

4.3. Toxicity by area

There was a significant difference between West Philippine and Sulu Seas ($p < 0.05$). This agrees with the previous study conducted by Montojo et al. (2020), stating that sampling sites' geographical

conditions significantly affect the occurrence of positive samples. In addition, toxicity was affected by the feeding mechanism of reef fishes. The movement of reef fishes from one area to another dramatically affects the presence of ciguatoxins. This only means that it was possible that fishes caught in a certain area may have accumulated these toxins from different localities that cause poisoning in people. For these reasons, varying toxicities in reef fishes between areas were possible.

The most recent outbreak of CFP was documented last 2014 in the Zamboanga City in Mindanao (PhilStar Global 2014; IOC WESTPAC 2016; Azanza et al. 2019). This was recorded after a year of conducting samplings in this study. The outbreak was implicated by *Sphyraena* species affecting 18 individuals. Before admission to a hospital, patients experienced nausea, loose watery stools, body malaise, abdominal pain, and numbness in extremities. Upon admission, abdominal pain, weakness, and moderate dehydration were observed from affected individuals. The most distinct symptom observed that distinguishes this type of poisoning is the hypersensitivity of the patients to hot and cold temperatures. Although the BFAR Field Office released a warning on the consumption of barracuda, people still consumed the fish.

4.4. Confirmation of CTX through symptoms

To the best of our knowledge, this was the first study in the Philippines that took the trouble to document the occurrence of CTX symptoms in mice and compare the symptoms with a particular CTX species (Pacific CTX-1). Contrariwise to the study of Pocsidio (1999), wherein the researcher performed histopathologic and physiologic tests on some experimental animal models proving that ciguatoxins adversely affect the internal organs of animals. It was crucial that symptoms after injection should be noted as several symptoms could indicate what type of toxin was present in the extracts. This was also the only way to characterize the observed toxins injected in mice since no reference standards were used in conducting this study. Based on the observed symptoms, FAO (2004) and EFSA (2010) suggest that the toxin present in the samples was Pacific CTX-1. Still, the researchers could not detect hypothermia since rectal temperature and pulse rate monitoring were not done. It should also be noted that negative samples also showed symptoms of ciguatera such as body weakness and wobbly upright gait but survived in the next following days. This may suggest that CTX was present but only in small amounts and not sufficient to kill the mice to be considered positive.

Since mouse bioassay was used in screening commercially important reef fishes, which was criticized by many scientific circles as having problems of non-specificity and insensitivity and not being able to distinguish between toxins (Botana et al. 2009; Campbell et al. 2011), the researchers suggest that more analytical methods must be used in characterizing and quantifying this type of toxin. In a study by Wong et al. (2014), they used mouse bioassay and LC-MS/MS methods in detecting CTX. Adoption of a two-tiered approach, chemical analysis (LC-MS/MS) for first-line screening of toxic fish coupled with biological assay (MBA) for confirmation, is considered an appropriate strategy, minimizing the use of laboratory mice and at the same time providing a rapid, sensitive, and reliable assay for screening potentially toxic fish. Additionally, Suzuki et al. (2017) also took this approach to detect ciguatoxins, which involves screening fish extracts using very sensitive functional assays. A second step involves confirming the presence of CTXs by liquid chromatography mass spectrometry (LC/MS).

5. CONCLUSION

Ciguatoxins are present in the West Philippine and Sulu Seas. This is observed from commercially important reef fishes such as *S. barracuda, S. quoyi, S. guttatus, P. leopardus, G. melanospilos,* and *L. campechanus*. *S. quoyi* had the highest toxicity of 0.65 ± 0.55 ppb and 0.48 ± 0.36 ppb found in flesh and viscera, respectively. All positive samples exceeded the guideline established by the US Food and Drug Administration and the Philippines' BFAR. Although higher toxicities were observed from fish viscera, toxicities between fish parts did not vary greatly. More sensitive and accurate methods are necessary for quantifying and characterizing these types of toxins. An analytical method using LC-MS/MS can accurately quantify and characterize ciguatoxins tandem with mouse bioassay as the screening method is a more effective approach than mouse bioassay alone. Establishing the methodologies in detecting CTX would greatly help monitor and manage CFP in commercially identified reef fishes in the country.

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AUTHOR CONTRIBUTIONS

Tanyag BE: Writing-Original Draft, Investigation, Methodology, Formal analysis, Writing-Review and Editing, Visualization. **Perelonia KBS**: Investigation, Formal analysis, Writing-Review and Editing. **Cambia FD**: Writing-Review and Editing, Supervision. **Montojo UM**: Conceptualization, Writing-Review and Editing, Project Administration, Funding Acquisition.

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS STATEMENT

The researchers followed all institutional and national guidelines for the care and use of laboratory animals.

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