Note

Incidence of ciguatoxin fish poisoning in Trivandrum, India

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ABSTRACT

Ciguatoxin (CTX) is a visibly unidentifiable, colourless, odourless, heat stable and lipid soluble polyether marine biotoxin associated with human illness. Marine dinoflagellates under the genus *Gambierdiscus* are responsible for producing ciguatoxins (CTX). The ciguatoxin gets accumulated in herbivorous fishes, gets biotransformed in carnivorous fishes and finally reach fish consumers. In January 2016, individuals who consumed red snapper in Trivandrum, Kerala, India were suspected to be intoxicated with ciguatera based on characteristic symptoms as assessed by medical team from Trivandrum Medical College, Kerala, India. The red snapper species was identified and confirmed as *Lutjanus bohar* by DNA barcoding. Mouse bioassay was carried out to detect the presence of ciguatoxin and the tested mice showed symptoms related to suspected CTX toxicity. Significantly higher level of ciguatoxin lethal dose was estimated which was found equivalent to 16.25 ng of CTX-1 which led to 13% of weight loss in tested mice. Medical professionals also investigated clinical manifestations of suspected toxicity in hospitalised individuals. This study indicated that there is a need for regular surveillance of seafood landed across the coast and consumer's awareness for their safety.

Keywords: Ciguatera fish poisoning, Ciguatoxin, *Lutjanus bohar*, Mouse bioassay

Ciguatoxin (CTX) affects approximately 50,000 to 500,000 people per year (Meyer *et al.,* 2015). Ciguatera fish poisoning (CFP) causes acute gastrointestinal and neurological symptoms, including vomiting, diarrhoea, abdominal pain, severe localised itching, tingling of extremities and lips, dysesthesia, as well as other chronic symptoms (Lewis, 2001; 2006). CFP occurs due to consumption of reef fishes of larger size in tropical and subtropical regions. More than 400 fish species are reported to be causative agents for ciguatera poisoning. Larger fishes accumulate gambiertoxins and their biotransformation in the fish makes it more potent in comparison to those in small fishes (Lehane and Lewis, 2000; Farrell *et al.,* 2016). The dinoflagellate species *Gambierdiscus toxicus* is the main source of the production of this marine toxin and its accumulation in fishes (FAO, 2004). Ciguatoxin is a colourless, odourless, heat stable and lipid soluble polyether compound. This toxin remains unaffected by freezing, drying or cooking process (Lewis, 2006; Abraham *et al.*, 2011). CTXs are secondary metabolites with numerous congeners having different molecular structure reported from different geographical areas such as Pacific, Caribbean and Indian regions (Caillaud *et al.,* 2010).

Incidence of CTX toxicity from Kerala coast has not been reported so far, as it had escaped many a times the attention of medical practitioners. The present study investigated a recent incident of food poisoning suspected due to ciguatera toxin from red snapper in Trivandrum, which presented characteristic symptoms in those who consumed the fish.

Fish samples for the investigations were collected from Vizhinjam, Kerala, India in January 2016, where intoxication in local population were reported. The poisoning occurred due to the consumption of cooked "red snapper" fish coming under the genus *Lutjanus*, purchased from the local fish market. Medical team from Trivandrum Medical College observed that the intoxicated persons had consumed same fishes and developed neurological and gastrointestinal complications similar to that of ciguatera fish poisoning. The fish samples collected were stored at -20^0C until analysis.

Caudal peduncle samples of the fishes were used for species authentication by sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene. The total genomic DNA from the samples was isolated using DNeasy Blood and Tissue Kit (Qiagen, Germany),

as per manufacturer's instructions and concentration as well as purity of the extracted DNA was estimated using a biospectrometer (Eppendorf, Germany). The COI gene was amplified using universal primer pair (Ward *et al.,* 2005) (Table 1).

Table 1. Primers used for identification of fish implicated in Ciguatera fish poisoning

Primer name	Sequence
Fish F1	5'- TCAACCAACCACAAAGACATTGGCAC -3'
Fish R1	5'- TAGACTTCTGGGTGGCCAAAGAATCA -3'

The reaction was carried out in 25µl volume, containing mixture of 1x taq buffer, 2.5 mM $MgCl_2$ and 50 µM of each primer, 200 µM of each dNTP, 0.5 U taq DNA polymerase, 75 ng of template DNA and autoclaved double distilled water to make up the volume to 25 µl. The reaction mixture was thermal cycled for 35 cycles of 30 s at 94°C, 30 s at 52°C, 45 s at 72°C and final extension of 10 min at 72°C, with an initial denaturation step at 95^0C for 4 min. The PCR products were sequenced bidirectionally using ABI 3730 capillary sequencer in the sequencing facility. The raw DNA sequences obtained were edited and aligned using BioEdit version 7.0.5.2 (Hall, 1999). The edited partial sequences of COI gene were analysed for species identification using the NCBI BLAST search engine and the sequences were submitted to GenBank database (NCBI, USA).

Extraction of ciguatoxin from fish samples was carried out as per IOC manuals and Guides No. 33 (Hallegraeff *et al.*, 1995) and European Union Reference method (ANSES, 2016) for mouse bioassay of ciguatoxin. Fifty gram of fish sample was cooked at 70^0C for 15 min, and cooled to room temperature. Tissue samples were then minced, diluted with acetone [3:1 V:W (ml g^{-1})] and homogenised for 5-15 min using a homogeniser (PRO Scientific Inc., USA) under iced condition. The homogenised samples were filtered using Whatman no.1 filter paper and the filtrate collected in a round bottom flask. Residual acetone and water were removed using a rotary evaporator (Heidolph, Germany) operated at 55^0C. The dried extract was transferred to a separatory funnel, added methanol:water (9:1), shaken well followed by extraction with 1:1 (v/v) n hexane and the upper hexane layer was discarded. This extraction process was repeated twice. The residual methanol:water was removed using vacuum evaporator. Further, ethanol:water (1:3) was added and shaken with diethyl ether (1:1) to separate the layers and the ether layer was collected. Ether extraction was repeated twice and ether fractions were pooled at an elevated temperature of 40-55^0C. The dried ether extract collected were assumed to contain the CTXs.

Ether extract was dissolved in chloroform:methanol (97:3) mixture and dried under N_2. The dried ether fraction was suspended in 1-5% tween 60/0.9% saline, sonicated for 180 s and filtered through 0.45 PTFE membrane filter prior to administering into mice.

Female albino mice weighing 20±2 g were used for the assay done in duplicate, by intraperitoneal injection with 0.5 ml of the prepared extract, whereas control mice were injected with only 0.5 ml tween 60/saline solution. Details such as time of injection, weight of mice, amount of extract (g) administered, time of onset and nature of signs and time of death were recorded for each injection. The post-injection behaviour was observed and recorded for at least 24 h. Weight loss in injected mice was also recorded at an interval of minimum 3 h duration.

The symptomatology in the hospitalised patients corroborated with earlier reports of ciguatera fish poisoning (Rajeish *et al.,* 2016) with typical clinical signs like gastrointestinal, neurological and cardiovascular symptoms. All six patients (one male and five females) were admitted with CFP symptoms of vomiting, diarrhoea, paraesthesia of upper limbs and lower limbs. Out of this, five patients belonged to one family comprising husband, wife and three daughters who live near Chakkipara Market, Trivandrum. Their symptoms started six hours after consumption of fish dish (chempalli curry). Symptoms like vomiting, diarrhoea, circumoral paraesthesia and paraesthesia of limbs were common to all family members. One of the three daughters also had paradoxical temperature reversal (cold objects sensed as hot and hot objects sensed as cold). All the patients were haemo-dynamically stable except the husband who had sinus bradycardia (low heart rate). The sixth patient was a female and her major symptom was giddiness. She also had abdominal pain and paraesthesia of limbs. In the affected individuals, the onset of ciguatera toxicity started within 24 h of consumption of fish curry and symptoms lasted for 1-4 days. However, in case of one individual, it persisted for six months as reported previously by Glaziou and Martin (1993) and Pearn (1995). Clinical diagnoses of CFP are reliable when a detailed and comprehensive history of the food source, onset of the illness and description of symptoms are accounted (Stewart *et al.,* 2009).

All the above said patients were treated by giving supportive measures like intravenous fluids and antiemetics. The husband's bradycardia improved and normal heart rate was restored after 2 days. Patients were in better condition at the time of discharge and the only symptom that persisted was paraesthesia of limbs.

The partial sequence of the mitochondrial CO1 gene from the tissue samples yielded an average length of 627 bp (Accession. No. KY057337). These sequences were used for identification of species based on the similarity search using the NCBI BLAST search engine and the species was identified as *Lutjanus bohar,* commonly known as two spot red snapper. *L. bohar* is a reef-associated tropical fish distributed along the Indo-Pacific region with earlier reports of ciguatera poisoning (Halstead *et al.,* 1990) and large fishes from oceanic areas in the western Pacific are often ciguatoxic (Dalzell, 1992). According to Oshiro *et al.* (2010), *L. bohar* weighing less than 4 kg to be non-toxic and 11.9% of the species exhibit CTX toxicity.

Intra-peritoneal injection of toxin extract from fish tissue induced symptoms in mice as indicated in case of CTX toxicity. A detailed description of symptoms of toxicity recorded in mice up to a 24 h period observation is given in Table 2. The prominent symptoms included piloerection, diarrhoea, lachrymation, dyspnoea, gasping, progressive hind limb paralysis, wobbly gait, terminal convulsions with tail arching, breathing difficulties, slow locomotor activity and hypothermia. However, these symptoms were absent when extract from control and negative (non-toxic) fish samples were administered in mice. The relationship between dose and time to death was used to quantify toxicity of the extract which ranged from 30 min to >10 h.

Traditional method of detecting the presence of ciguatoxin in fish involves testing lipid extracts by mouse bioassay (Lewis and Sellin,1993) and the most widely used mouse bioassay method was described by Yasumoto *et al.* (1984) which has been accepted worldwide. The lethal dose *i.e.,* LD_{50} dose for a 20 g mouse is equal to one Mouse Unit (MU) which is equivalent to 5 ng CTX-1. One MU is equivalent to 5 ng, 18 ng and 48 ng for Pacific CTXs, P-CTX-1, P-CTX-2 and P-CTX-3, respectively (Lewis *et al.,* 1991; Lewis and Sellin, 1993) and 72 ng for pure Caribbean CTX-1 (Pottier *et al.,* 2003). Dose and time to death relationship for a mix of ciguatoxins typically found in carnivorous fish is defined according to the equation:

$$\text{Log MU} = 2.3 \log (1 + 1/T)$$

where, T is the time to death in hours (Lewis and Sellin, 1992). The lethal dose was estimated to be 3.25 MU per 20 mg of ether extract and the amount of CTX toxicity in fish sample is equivalent to 16.25 ng of CTX -1, which is significantly higher than the reported levels of CTX intoxication in humans. It was formerly suggested that any fish containing above 2.5 MU 100 g^{-1} should be avoided as food (Yasumoto *et al.,* 1984) since ciguatoxins are potent neurotoxins that may have long-term neurological effects. The average weight loss observed in the positive sample was calculated as 13%. The evaluation of toxicity of the fish tissue samples based on mouse bioassay was interpreted as shown in Table 3. The death of 1 or 2 mice within 24 h is interpreted as positive for ciguatoxicity and the fish sample is rated as inedible. In the absence of death, weight loss >5% after 24 h of injection of atleast one mouse is considered as a positive result for ciguatoxicity and the fish sample is considered as edible to limited extent. When there is no mortality or weight loss, then the sample is edible without doubt. MBA (mouse bioassay) provides a measure of total toxicity based on the biological response of the animal to the toxins but no specific information is provided on individual toxins.

Table 2. Symptoms of ciguatera toxicity recorded during mouse bioassay

Symptoms	Evaluation	Animal responses	
		Control	Toxin treated
Hypothermia	Thermometer	35 - 38^{0}C	Below 33^{0}C
Piloerection	Observation	None	Mild to marked
Lachrymation	Observation	Normal	Mild to severe
Hyper salivation	Observation	Absent	Mild to severe
Dyspnoea	Observation	Absent	Mild to severe
Wobbly upright gait	Observation	Absent	Present
Gasping	Observation	Absent	Mild to marked
Withdrawal reflex	Grasp hind leg	Withdrawal	Reduced to absent
Mild gasping	Observation	Absent	Present
Diarrhea	Observation	Absent	Mild
Breathing difficulties	Observation	Absent	Rapid shallow to intermittent gasping
Locomotor activity	Observation	Normal	Slow to absent
Hind limb paralysis	Observation		Progressive paralysis from hind limb extending to fore limbs
Convulsions	Observation	Absent	Tonic and/or Jumping

Table 3. Interpretation of ciguatoxicity of fish tissue samples based on mouse bioassay

Test sample	No. of dead mouse (s) in 24 h	Weight loss >5% after 24 h injection	Conclusion
Suspected sample 1	One of two	Yes	Positive, Not edible
Suspected sample 2	0	Yes	Positive, limited edibility
Control	0	No	Negative, edible

Source: EU-NRL (ANSES, 2016)

Banner *et al.* introduced the MBA for CTXs in1960 and this is most widely used mammalian *in vivo* model for toxicity screening of CTXs (Caillaud *et al.,* 2010). Along Indian coast, CFP incidents are infrequent. Routine analysis of samples by mouse bioassay cannot be recommended since it is non-specific and ethically objectionable (Abraham *et al.,* 2011) and considered as a toxicological tool accessible only to selected laboratories. We carried out mouse bioassay test to identify and quantify CTX toxins in order to provide further support for the clinical diagnosis of the CFP incident. Proficiency in the ability to identify the toxic fish and effective clinical recognition will definitely improve our understanding of the source of poisoning.

This study is the first report in incidence of ciguatoxin poisoning along Kerala coast and signifies the importance of seafood safety. In the absence of commercial testing, a precautionary approach is necessary for the surveillance of CTX intoxication along the Indian coast. The implementation of regulatory criteria for CTXs would be needed with respect to aspects like identification of ciguatoxic fish mainly reef associated fish, regulatory measures such as ban or size restrictions on high-risk species and misdiagnosis or under-reporting of CFP cases. A rapid and reliable instrumentation method through mass spectrometry, with the aim of routine monitoring and screening of CFP in reef fishes along the Indian coast is the need of the hour. The study also signifies the need for creating awareness regarding consumption of coral reef fishes and its consequences among the public.

Acknowledgements

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