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Research Note

Confirmation of Ciguatoxin Fish Poisoning in Red Snapper, *Lutjanus bohar* (Forsskål, 1775) by Mouse Bioassay

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Ciguatoxin is an important biotoxin in entering the human body through the consumption of coral reef fishes. The biotransformation of Gambiertoxins in large fishes makes it more potent and significant in respect of human health (Friedman et al., 2017; Dickey et al., 2010; Lehane & Lewis, 2000). A benthic dinoflagellate known as *Gambierdiscus toxicus*, is responsible for the production of Gambiertoxin. Ciguatoxin, a tasteless, colourless, odourless, heat and acid stable, lipophilic polyether compound which is stable at freezing temperature also (Abraham et al., 2011). CTXs secondary metabolites with numerous congeners having different molecular structure have been reported from different geographical origins namely Pacific (P-CTX), Caribbean (C-CTX) and Indian (I-CTX) (Caillaud et al., 2010). This region-specific biotoxin has been reported very recently from Mangalore coast (Rajeish et al., 2016). Earlier in January 2016 the same fish species detected with ciguatoxic from Vizhinjam coast which had caused toxicity in local population were reported by Rajisha et al. (2017). The existence of ciguatoxicity has not been indicated by any highly visible surface phenomenon such as red tide as seen in the case of Paralytic Shellfish Poisoning (de Fouw et al., 2001), hence an early warning to the alarm of CFP incidence is not possible. Studies indicate that the number of people affected by ciguatoxin fish poisoning (CFP) ranged from 10000 to 50000 (Lewis, 2001; Caillaud et al., 2010) mainly in the tropical and sub-tropical regions of the world. This shows the intensity of occurrence even though it is difficult to ascertain the under-reporting cases of CFP (Friedman et al., 2017). A wide variety of 400 finfish species are found to elaborate ciguatoxin (Lewis, 2006) and it is responsible for the substantial economic loss because of the chronic health impacts after fish consumption. Mouse bioassay (MBA) has been widely used for the selective determination of ciguatoxicity in fishes (Banner et al., 1960).

A total of 262 reef associated finfish samples were collected from different sources across Kerala, Tamil Nadu, Maharashtra, Gujarat and Karnataka for the determination of ciguatoxicity using MBA. Screened samples for ciguatoxicity comprised of four species of snappers *viz., Lutjanus argentimaculatus, L. fulvus, L. bohar and L. gibbus;* two species of Barracuda *viz., Sphyraena putnamae and S. jello;* four species of reef cod *viz., Epinephelus bleekeri, E. coioides, E. diacanthus* and *E. chlorostigma;* other species include *Pristipomoides filamentosus, Otolithoides biauritus, Caranx ignobilis, Lethrinus nebulosus, Variola louti, Aprion virescens etc.* DNA barcoding based on COI gene is employed for species-level fish identification using DNeasy Blood & Tissue Kit (Qiagen, Germany). The raw DNA sequences obtained by sequencing were edited and aligned using Bio Edit version 7.0.5.2 (Hall, 1999). Mouse bioassay of fish ether extracts was used for toxin analysis (ANSES, 2016; Lewis. 1995). Fish sample was extracted by acetone from 100 g of cooked (70°C) and minced tissue homogenate. After evaporation of acetone, the residue was dissolved in methanol-water mixture (9:1) and liquid-liquid partition with n-hexane allowed the removal of non-polar lipids. The residue obtained after evaporation of methanol phase was taken up in ethanol-water (1:3) and separated with diethyl ether. Ether phase evaporated and dissolved in chloroform: methanol (97:3) and residue collected after this phase is reconstituted in 1% 0.5 mL Tween 60/0.9% saline and filtered through 0.45 PTFE

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membrane filter prior to administering (intra peritoneal) into mice. Female albino mice weighed around 20±2 g was taken for analysis. Control mice was injected 0.5 mL 1% Tween 60/0.9% saline alone.

Fish samples were partially sequenced which yielded an average length of 641bp for cytochrome oxidase subunit I (COI) gene (Accession No. MF383185). A BLAST analysis of COI sequences showed 100% similarity to *L. bohar*. Phylogenetic tree (Fig. 1) for the COI sequences with other Lutjanus sp. showed it to be closest to *L. bohar.* Species identification through molecular taxonomic techniques provides a better understanding of the species. Two fish samples of *Lutjanus bohar* (weighed 6.39 and 5.62 kg respectively) collected from Thoppumpady (Kerala) and Mangalore (Karnataka) in October 2016, were confirmed for ciguatoxicity using Mouse bioassay method (ANSES, 2016; Lewis, 1995). A check list (Table 1) of symptoms observed during experiment was prepared for up to 24 h after intra peritoneal injection in to mice. Symptoms started with reduced locomotor activity, diarrhea within 1 h of intra peritoneal (i.p) injection of fish extract. The death of one or two mice within 24 h is interpreted as positive and it is confirmed that the fish were not fit for human consumption. Symptoms observed included progressive paralysis (hind limb), breathing difficulties, convulsion (body muscles contract and relax rapidly), stretching of hairs in an erected manner (piloerection) etc. within 4 h of administration of extract. This extraction method allows the quantification of ciguatoxin in fish flesh up to 20 mg of ether extract. The lethal dose i.e., LD_{50} dose for a 20 g mouse is equal to one Mouse Unit (MU) which is equivalent to five Nano

Table 1. IOC Mouse bioassay for Ciguatoxin (Check list)

Sl No.	Symptoms	1st h	2nd h	3rd h	4th h	24th h
1.	Hypothermia	+	+	+	ND	ND
2.	Hypothermia below 33°C	+	+	+	ND	ND
3.	Piloerection	+	+	+	ND	ND
4.	Diarrhoea	+	+	+	ND	ND
5.	Lachrymation	+	+	+	ND	ND
6.	Hyper salivation	+	+	+	ND	ND
7.	Dyspnoea	+	+	+	ND	ND
8.	Wobbly upright gait	+	+	+	ND	ND
9.	Gasping	+	+	+	ND	ND
10.	Mild gasping	+	+	+	ND	ND
11.	Terminal Convulsion with tail arching	+	+	+	ND	ND
12.	Hind limb paralysis	+	+	+	ND	ND
13.	Progressive hind limb paralysis	+	+	+	ND	ND
14.	Progressive paralysis from hind extending to fore limbs	+	+	+	ND	ND
15.	Convulsions	+	+	+	ND	ND
16.	Mild Convulsions preceding death>30 sec	+	+	+	ND	ND
17.	Respiratory problems	+	+	+	ND	ND
18.	Respiratory failure	+	+	+	ND	ND
19.	Death from respiratory failure	+	+	+	ND	ND
20.	Slow Movements	+	+	+	ND	ND
21.	Slow locomotor activity	+	+	+	ND	ND
22.	Breathing Difficulties	+	+	+	ND	ND
23.	Sluggish	+	+	+	ND	ND

Note: ND – Not Detected as mice death occurs within 3-4 h from the time of injection.

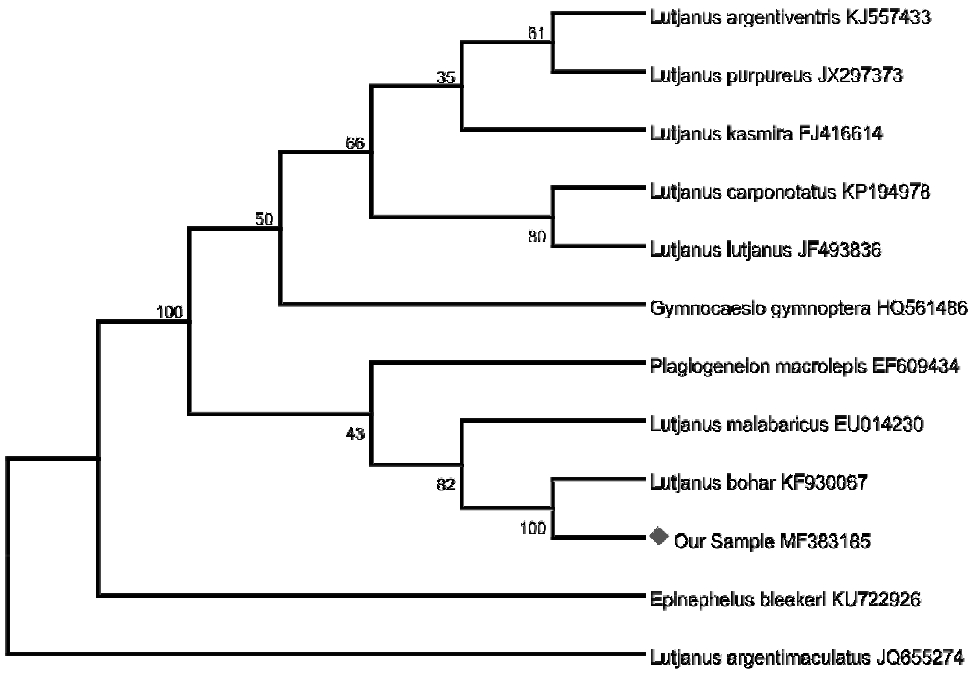

Fig. 1. Phylogenetic tree of Lutjanus bohar generated based on partial (641bp) COI gene sequences.

gram CTX (Lewis, 1995). Dose and time to death relationship for a mix of ciguatoxin typically found in carnivorous fish are defined according to the equation: log MU = 2.3 log (1 + 1/T), where T is the time to death in hour (Lewis, 1995). The toxic fraction suspended in 0.5 ml 1% Tween 60/0.9% saline and assayed in duplicate. As the death time calculated during the experiment is 2.67 h, the lethal dose estimated to be 2.08 MU 20 mg^{-1} of ether extract according to the equation and the amount of CTX toxicity in fish sample is equivalent to 10.4 ng of CTX -1.

Ciguatoxin sample analysis data based on IOC manuals and guides mouse bioassay method (Lewis, 1995) has been submitted as an agenda item 3 in Codex Committee on Contaminants in foods, eleventh session (CCCF, 2017). In the absence of commercial testing, a reliable laboratory analytical method is needed to confirm the presence of ciguatoxicity among the reef fish samples meant for human consumption. Fish carrying ciguatoxin do not exhibit any symptoms and it is practically difficult to ascertain whether the fish is toxic or not. Existence of CTXs along Indian coast calls for a need for good surveillance system and analytical confirmatory methods for the protection of consumers along with exporters. Hence mouse bioassay of fish extract considered as a reliable approach to detect the presence of sub lethal doses of CTXs through intermittent observation of symptoms for up to 48 h (Caillaud et al., 2010). In the present scenario, where food safety is becoming the prime concern e, it is felt that the ciguatera poisoning will became a major concern in the marketing of reef associated fin fishes. The amount of toxin is directly correlated to the size of the fish and results indicated that large sized fishes had more ciguatoxin in comparison to small fishes (Pottier et al., 2001). Hence it is advisable for the consumers to take only *L. bohar* of small size. Ban or size restrictions on certain reef fish species can be taken as an initial safety measure to protect the consumers from the lethal effects of this toxicity.

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