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Presumptive case of ciguatera fish poisoning in Mangalore, India

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Ciguatera fish poisoning (CFP) occurs when humans ingest fishes contaminated with ciguatoxins (CTXs). Two individuals developed suspected ciguatera poisoning after consuming unknown fish purchased from a local market in Mangalore, India. DNA barcoding confirmed the fish under study to be *Lutjanus bohar*. A mouse bioassay study detected high levels of CTX in the implicated fish. Mice injected with toxin showed typical symptoms of CTX poisoning. To the best of our knowledge, this is a first case report of CFP due to consumption of *L. bohar* in India.

Keywords: Ciguatera fish poisoning, cytochrome oxidase, DNA barcoding, *Lutjanus bohar*, mouse bioassay.

CIGUATERA FISH POISONING (CFP) is the human intoxication caused by consumption of fish which have accumulated ciguatoxins (CTXs). Ciguatoxin is a colourless, odourless, heat stable, lipid-soluble polyether which is not destroyed by the cooking process[1]. Ciguatoxin is primarily produced by dinoflagellates of the genus *Gambierdiscus*. Herbivorous fish that graze on these dinoflagellates bio-accumulate the toxin, which then gets passed on to predatory fish via the marine food chain and finally to humans[2]. Distribution of ciguateric fish is restricted to tropical and subtropical waters, being particularly common in Pacific and Indian oceanic regions and in the tropical Caribbean Sea[3]. Depending on their geographical origin and chemical structure, CTXs are classified as Pacific (P-CTX), the Indian Ocean (I-CTX) and the Caribbean (C-CTX) of which P-CTXs are considered to be the most potent[4,5]. CFP outbreaks outside of endemic areas have been attributed to consumption of imported toxic fish[6] and expanding biogeographical range of *Gambierdiscus* spp. and ciguatoxic fish[7,8].

CFP symptoms vary with the regional origin of the toxin[9] and occur within 0.5–12 h of toxic fish consumption[10]. Clinical presentation of CFP is characterized by a range of gastrointestinal symptoms that include abdominal pain, nausea, vomiting and diarrhoea, usually followed by neurological symptoms such as pruritus (itchy skin), dysesthaesia (reversal of hot and cold sensations), numbness and tingling in the extremities, paresthesia, ataxia, and

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also cardiovascular symptoms[11], which could last from few days to several months[12]. The symptoms are aggravated by alcohol consumption, tobacco smoking and fish consumption[11].

It is difficult to recognize the toxin in ciguateric fish as it seems to be insensitive to the toxic effects and is devoid of changes in texture, smell or taste as compared to non-toxic fish[11]. Several coral reef fishes (>400 species) have been reported to be carriers of ciguatera[12]. The incidence of ciguatera disease is estimated to be 50,000 annually around the world despite large cases of under-reporting[13]. Significantly different forms of symptoms may exist between patients and patients from distinct geographical areas may present different symptoms[14]. Some researchers have suggested the intravenous injection of mannitol as the therapy for chronic CFP[15].

In June 2015, two individuals of a family aged 60 and 30 years became ill following consumption of cooked fish and were hospitalized. The consumer had purchased the fish from a local market in Mangalore. The fish name was unknown to the consumer as it was sold as fish remnants after filleting of fish. The fish purchased was mainly the head and meat portions left over after filleting. A portion of the fish, mainly the head, was used in 'curry' preparation and the remainder refrigerated. The affected individuals complained of gastrointestinal and neurological symptoms exactly 4 h after fish consumption. They were hospitalized with symptoms such as chest burning, abdominal pain, vomiting, diarrhoea, pruritus of legs and hands, tingling sensation in throat and tip of the tongue, paresthesia of the extremities, arthritis, difficulty to walk, weakness and cold allodynia. The patients were treated with mannitol, considered to be an effective antidote and medicine for symptomatic treatments. The symptoms in the younger individual was less severe and was discharged after a day of stay in hospital. However, in the older affected individual, although the initial gastrointestinal symptoms had subsided, he complained of feeling hot–cold sensation in hands and feet even after two months of treatment, a characteristic symptom of ciguatera poisoning[14].

The unknown fish sample in frozen condition was received from affected consumers who had suffered from suspected fish poisoning. The frozen raw fish procured from the patient was immediately stored at –20°C in the laboratory for further analysis.

Fish received at the laboratory was in chopped condition and therefore identification of the fish was difficult. We therefore subjected the available fish sample to DNA barcoding for its identification. Total genomic DNA was extracted following the method described by Sambrook[16] with minor modifications. The final concentration of the DNA obtained was 500 ng/µl (NanoDrop, USA).

The DNA extracted was subjected to PCR amplification of the cytochrome oxidase subunit I (COI) and 16S rRNA genes for identifying the unknown fish. A 684 bp fragment of COI was amplified using the universal primer FishF1 : 5′-TCAACCAACCACAAAGACATTGGCAC-3′ and FishR1 : 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′ (ref. 17). Similarly a 593 bp fragment of the 16SrRNA was amplified using the primer pair 16S F : 5′-CGCCTG-TTTATCAAAAACAT-3′ and 16S R : 5′-CCGGTCTGA-ACTCAGATCACGT-3′ (ref. 18). The PCR reactions were performed in 30 µl reaction volume, containing 3 µl of 10X buffer (100 mM *Tris*-HCl, 500 mM KCl, 50 mM $MgCl_2$), 2.4 µl of dNTPs (2.5 mM), 2 µl of each forward and reverse primers (2 µM), 2 µl of template DNA, 0.5 µl of *Taq* DNA polymerase (0.9 U) and 18.1 µl of ultrapure water. Cycling parameters consisted of an initial denaturation step of 4 min at 95°C, followed by 35 cycles of 1 min at 95°C, 30 sec annealing at 62.5°C and 62°C for COI and 16S RNA gene, respectively and 1 min extension at 72°C, with a final extension step of 15 min at 72°C. The PCR reactions were carried out in a thermocycler (MJ Research, USA). The amplified PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized using the Geldoc system (BioRad, USA). Each target gene was amplified in three independent reactions from triplicate DNA samples of the fish. The PCR products were purified using a Roche PCR cleanup kit (Mannheim, Germany) and sent for sequencing (Bioserve Biotechnologies, Hyderabad, India).

The identity of the experimental nucleotide sequences to known sequences in GenBank was performed using the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov). The COI and 16S rRNA gene sequences in this study showed highest identity to corresponding gene sequences of *Lutjanus* spp. in GenBank. Partial sequences of mitochondrial COI and 16S rRNA gene sequences pertaining to *Lutjanus* sps. were downloaded from NCBI. Multiple sequence alignment was carried out using the ClustalW program[19]. Phylogenetic tree was generated by the neighbour-joining method using MEGA 4.0 (ref. 20).

Fish samples were subjected to ciguatera toxin extraction following the method of Lewis[21]. Briefly, 100 g of the fish sample was thawed and cooked at 70°C for 15 min in a plastic bag and cooled to room temperature. On cooling, sample was minced and homogenized with acetone (3 l/kg flesh) for 15 min. The fine slurry was vacuum filtered using Whatman #1 paper and acetone solubles collected. The extract was then dried to viscous slurry on a rotary evaporator at 55°C. The extract was re-dissolved in 90% of aqueous methanol (0.5 l/kg flesh) and extracted twice with hexane (1 : 1, v : v) in a separatory funnel. The lower aqueous methanol layer was dried in rotary evaporator at 55°C and re-dissolved again in ethanol–water (1 : 3) and extracted with diethyl ether (1 : 1, v : v) thrice in a separatory funnel. The top ether layer was collected and diethyl ether extract was concentrated in rotary evaporator at 55°C, re-dissolved in a known volume of chloroform–methanol (97 : 3, v : v) for

quantification and dried under a stream of N_2. The toxin extract was stored in –80°C until further use.

The toxin extract was tested for potential presence of CTXs and its toxicity by mouse bioassay as previously described[21]. Briefly, BALB/c mice weighing 18–22 g were obtained and housed in a controlled-environment at ~25°C and fed with food and water. Mouse bioassay was performed using a portion (~20 mg/mouse) of the diethyl ether fraction. The fraction was suspended in 0.5 ml 1% Tween 60/0.9% saline, heated at 37°C until dissolved, cooled and injected intra-peritoneally into healthy mice in duplicates. Control mice were administered with 0.5 ml of 1% Tween 60/0.9% saline. The mice were observed for symptoms of CTX poisoning and their survival time recorded. The relationship between dose and death time was used to quantify each fraction. The quantity of CTX present was calculated by: $\log MU = 2.3\log(1 + T - 1)$, where MU = number of mouse units of CTX injected and T = time to death in hours. One MU is the lethal dose for a 20 g mouse, which is equivalent to 5 ng CTX-1[22].

Fish identification is traditionally based on morphological characteristics. However, in recent years, 'DNA barcoding' wherein short DNA sequences are used in species identification has become increasingly popular. In fisheries, DNA barcoding has found application in determining the taxonomic identity of unknown fish species, damaged fishes and mislabelled fishery products[23]. The genes commonly recommended in DNA barcoding are the cytochrome C oxidase 1 (COI-1) and the 16S rRNA gene[24]. Thus, we targeted the 16S rRNA and COI mitrochondrial genes for DNA barcoding and identification of fish sample received at the laboratory. The primers reproducibly generated single amplification products with an average length of approx. 684 bp for COI-1 and 593 bp for 16S-rDNA (Figure 1). The amplified products were sequenced and deposited in GenBank with accession numbers KU050105 for COI and KU050104 for 16S rRNA.

A BLAST analysis of both COI and 16S rRNA sequence showed closest identity to *Lutjanus* sps. Phylogenetic analysis for the COI (Figure 2) and 16S rRNA (Figure 3) gene sequences together with respective sequences of other known *Lutjanus* sps. showed it to be closest to *L. bohar* and thus we presume the unknown fish in this sample to be probably *L. bohar*.

L. bohar is a coral reef inhabitant, being found at depths from 4 to 180 m, though usually between 10 and 70 m (ref. 25). This species is native to the Indian Ocean, but is widespread in the Indo-Pacific from the east African coast to the western Pacific Ocean, north to the Ryukyu Islands, and south to Australia[25]. Since the first isolation and characterization of CTX from *L. bohar* and *L. sebae*[26], several studies have successfully implicated *L. bohar* as the vector of I-CTXs[27]. *L. bohar* (two-spot red snapper) is reported to be the most common reef fish implicated in the CFP poisoning in Hong Kong[28] and Okinawa, Japan[29]. Further, toxicity tests using mouse bioassay have reported that 11.9% of *L. bohar* fishes are ciguatera toxic with individuals weighing less than 4 kg to be non-toxic[29].

In this study, based on patient symptoms and subsequent reference to literature, we presumed the toxicity symptoms to be related to that of ciguatera poisoning. The mouse bioassay method described by Lewis[21] is currently the most widely used assay for the detection of ciguatoxins in fish, wherein the relationship between dose and time to death is used to quantify toxicity. We performed the mouse bioassay to establish the potential

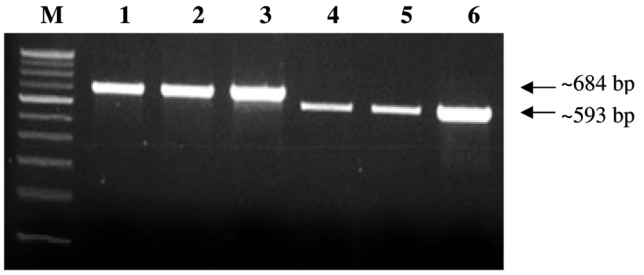

Figure 1. PCR amplification of the COI and 16S-rDNA gene in fish sample. Lanes M: marker (100 bp ladder), lanes 1–3: Bands corresponding to COI gene fragment, lanes 4–6: Bands corresponding to 16S RNA gene fragment.

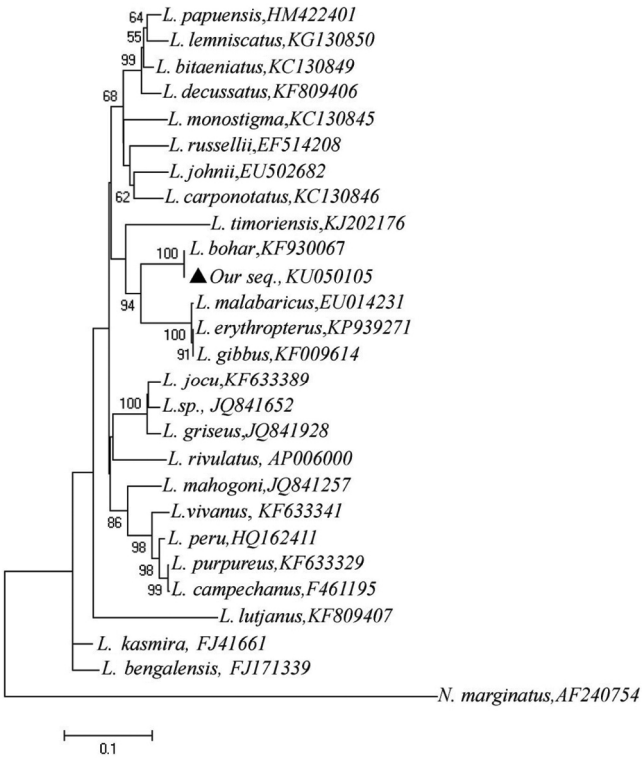

Figure 2. Neighbour-joining tree of *Lutjanus* species generated based on partial (575 bp) COI gene sequences. The bootstrap values >50% inferred from 1000 replicates are shown next to the branches. *N. marginatus* COI gene sequence (AF240754) was used as an outgroup. ▲ refers to COI gene sequence from this study.

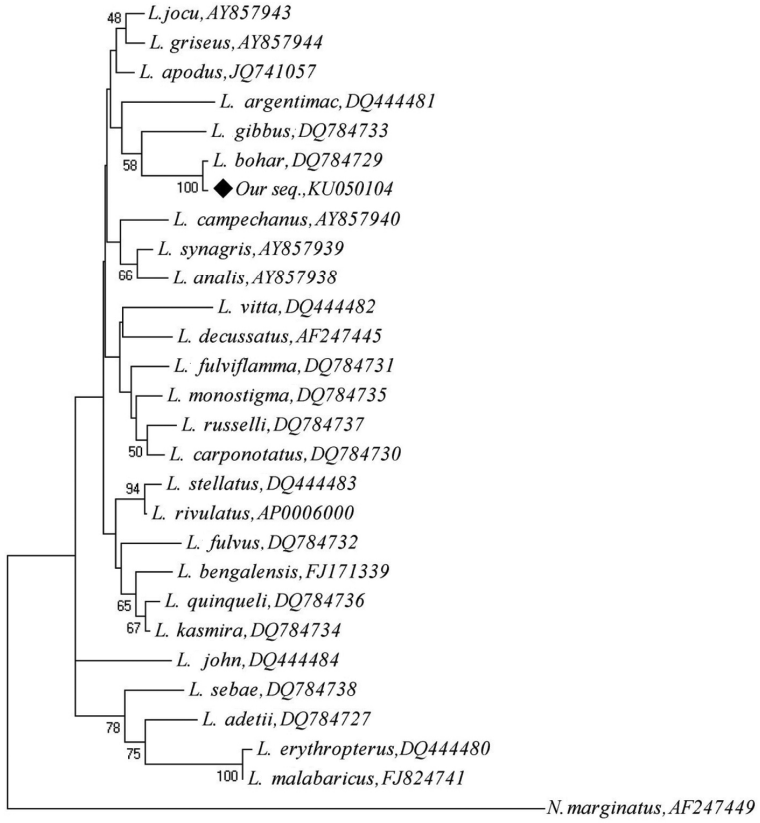

Figure 3. Neighbour-joining tree of *Lutjanus* species generated on basis of partial (540 bp) 16S rRNA gene sequence. The bootstrap values >50% inferred from 1000 replicates are shown next to the branches. *N. marginatus* 16S rRNA gene sequence (AF247449) was used as an outgroup. ◆ refers to 16S rRNA gene sequence from this study.

presence of CTXs in the fish sample and to quantify it. The toxin injected mice exhibited typical symptoms of ciguatera poisoning such as reduced locomotor activity, severe diarrhoea, hind limb paralysis, gasping for air, breathing difficulty and finally death within 2 h. However, these symptoms were absent in control mice. The lethal dose was estimated to be 3.4 MU/20 mg of ether extract and the CTX in fish quantified to be equivalent to 17 ng of CTX-1. The mouse toxicity of cooked and raw fish to cause intoxication has been reported to be 0.1 MU/g (= 0.7 ng CTX-1/g flesh) and 0.05 MU/g (=0.35 ng CTX-1/g) respectively[29]. The level of CTX-1 observed in this study was considerably higher than the levels reported to bring about symptoms of ciguatera poisoning in humans. Ciguatoxins are reported to be more concentrated in fish head, viscera, roe and skin[30]. According to the information gathered in this study, the older affected individual had consumed more of the fish head which probably we presume could have been the reason for the severity of gastrointestinal and neurological symptoms experienced by him.

This study is the first to report an incidence of CFP in India. Though India has vast maritime area and consumes marine fishes in abundant quantity, there is paucity of data on cases concerning ciguatera fish poisoning. This could be probably due to lack of awareness about toxic fish or symptoms of CFP among public, avoiding consulting doctors or lack of knowledge among health professionals to recognize the disease. Since the ciguateric fish cannot be distinguished on the basis of its taste, odour or appearance, the public in large are at the risk of poisoning due to consumption of such fishes[11]. Therefore, a good surveillance system, a reliable and reproducible laboratory test for identification of ciguateric fish, regulatory measures such as bans on high risk fish[12] is required to protect public from ciguatera poisoning. It has also been suggested that as a precautionary measure, consumption of coral fishes >1.0 kg should be avoided to prevent CFP[31].

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