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Ciguatera incidence and fish toxicity in Okinawa, Japan

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ABSTRACT

Okinawa being located in the subtropical region has the highest incidence of ciguatera in Japan. Officially, 33 outbreaks involving 103 patients have been reported between 1997 and 2006. The implicated species were *Variola louti*, *Lutjanus bohar*, *Lutjanus monostigma*, *Epinephelus fuscoguttatus*, unidentified *Lutjanus* sp., *Plectropomus areolatus*, *Oplegnathus punctatus*, *Epinephelus polyphekadion*, *Caranx ignobilis* and moray eel. Toxicities of the leftover meals, as determined by mouse bioassays, ranged from 0.025 to 0.8 MU/g or above (equivalent to 0.175–5.6 ng CTX1B/g). We collected 612 specimens of fish belonging to *L. monostigma*, *L. bohar*, *Lutjanus argentimaculatus*, *Lutjanus russellii*, *V. louti*, *Variola albimarginata*, and *E. fuscoguttatus* from the coasts around Okinawa and examined the toxicity of the flesh by the mouse bioassay. The rate of toxic fish was as follows: *L. monostigma*: 32.3%, *L. bohar*: 11.9%, *V. louti*: 14.3%, *E. fuscoguttatus*: 20.8%. Only one out of 36 samples of *V. albimarginata* and two of 74 samples of *L. russellii* were found toxic. None of the 35 samples of *L. argentimaculatus* was toxic. Nor the *L. bohar* samples weighing less than 4 kg were toxic. In all toxic samples, CTX1B was detected by LC/MS analysis but CTX3C and 51-hydroxyCTX3C were not.

1. Introduction

Ciguatera fish poisoning (CFP) associated with gastrointestinal, cardiovascular and neurological symptoms and signs is one of the largest scale food poisonings of nonmicrobial origins annually affecting 20,000–60,000 people worldwide (Lehane and Lewis, 2000; Yasumoto, 2005). While ciguatera endemic areas are tropical and subtropical Indo-Pacific Ocean and Caribbean Sea where coral reef developed, increasing world trade of seafood and international tourisms cause outbreaks in other climate (Lehane and Lewis, 2000; Yasumoto, 2005; Wong et al., 2005). Original forms of principal toxins, ciguatoxins (CTXs), are produced by an epiphytic dinoflagellate *Gambierdiscus toxicus* and transferred to herbivorous and carnivorous fish via food chain (Yasumoto et al., 1977; Yasumoto, 2005). The chemical structures of ciguatoxins in *G. toxicus* and in fish have been elucidated (Yasumoto, 2001, 2005). Ciguatoxin-4A (CTX4A) and ciguatoxin-3C (CTX3C) produced by the dinoflagellate were presumed to undergo structural changes in fish to produce the most representative toxin, ciguatoxin-1B (CTX1B), 51-hydroxyCTX3C and other metabolite toxins (Yasumoto, 2001, 2005). Occurrence of

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CFP is rather rare in Japan, and Okinawa is the only area where sporadic but regular occurrence of CFP is reported. Okinawa is the largest of the Ryukyu archipelago that consists of 48 islands lying in the south-westernmost end of Japan. Its climate is subtropical and CFP has been recognized as endemic in a previous survey (Hashimoto et al., 1969a,b).

In this paper, we summarized CFP outbreaks occurred in Okinawa, analyzed fish toxicity, and demonstrated the presence of CTX1B.

2. Materials and methods

2.1. Epidemiological data

CFP incidents officially reported by medical doctors to the Department of Health and Welfare, Okinawa Prefectural Government, between 1997 and 2006 are used for epidemiological analysis in this paper. The toxic levels of implicated fish were determined by the official mouse bioassay method (MBA, Satake, 2005), whenever the left-over food and/or remnant fish were available.

2.2. Chemicals

Normal saline solution (0.9% NaCl) was purchased from Otsuka Pharmaceuticals Co., Ltd.(Tokyo). The HPLC grade methanol, distilled water, and formic acid obtained from Wako Pure Chemical Industries, Ltd.(Tokyo), were used for the mobile phases in LC/MS analysis. Standard toxins, CTX1B, CTX3C and 51-hydroxyCTX3C for LC/MS were synthesized at Tohoku University (Hirama et al., 2001; Inoue et al., 2006). Other chemicals used were of analytical grade, otherwise stated. The composition of a solvent mixture was expressed in v/v.

2.3. Fish samples

Frozen fish specimens of *Lutjanus monostigma*, *Lutjanus bohar*, *Lutjanus argentimaculatus*, *Lutjanus russellii*, *Variola louti*, *Variola albimarginata*, and *Epinephelus fuscoguttatus*, collected from the Okinawan coasts were purchased from the Fishermen's Unions and stored at −20 °C until use. The fish were thawed, photographed, measured for standard length and weight, dissected into flesh, liver, and other viscera, and frozen again at −20 °C until use. Professor Tetsuo Yoshino, University of the Ryukyus, kindly conducted identification of the fish species.

2.4. Extraction

Fish samples were extracted following the standard MBA method for ciguatoxin detection. A flesh sample (120 g) was thawed and homogenized with 350 ml of acetone twice. The combined acetone extract was evaporated to produce an aqueous concentrate, which was further extracted with 100 ml of diethyl ether twice. The ether extract was evaporated completely and the residue was partitioned between hexane (50 ml) and methanol–water (9:1, 25 ml). The aqueous methanol layer was completely freed of methanol and the residue was used for the mouse bioassay. In all experiments, evaporation of a solution was carried out under reduced pressure.

2.5. Mouse bioassay (MBA)

The standard MBA method was slightly modified to reduce the number of mice for use. The residue from an extract equivalent to 120 g of the flesh was suspended in 3 ml of 1% Tween 60 in normal saline solution. Two 1-ml portions of the suspension were injected respectively into a male mouse of ddY strain weighing 17–20 g by intraperitoneal route (Kyudo co., Ltd., Kumamoto, Japan). The mice were observed for 24 h. When the two mice died or survived, the sample was regarded as toxic or nontoxic, respectively. If one was dead and the other survived, remaining 1 ml of the sample suspension was injected into another mouse. The toxicity of the sample was judged by the survival or death of the third mouse.

The flesh samples judged to be toxic by MBA were extracted similarly. Serial dilutions of the extracts were prepared and used for further confirmation of the toxicity levels by MBA. For example, a suspension equivalent to 20 g flesh/ml was prepared and 1 ml and 0.5 ml portions were injected, then the suspension was diluted four times with Tween 60 solution and used as same manner.

2.6. Solid phase extraction (SPE) and LC/MS analysis

An extract prepared from 20 g of flesh was dissolved in 20 ml of methanol–water (7:3) containing 1% ammonium hydroxide. The solution was applied to an OASIS HLB cartridge (6 cc, 200 mg, Waters, Massachusetts) preconditioned with methanol followed by distilled water. The toxins retained on the cartridge were eluted first with 3 ml of methanol–water (8:2) containing 1% ammonium hydroxide (Fr. A) and next with 2 ml of acetonitrile–water (7:3) (Fr. B). Fractions A and B were dried and dissolved in 0.5 ml of methanol. A 0.1 ml portion of the each solution was mixed with 0.9 ml of chloroform and passed through a silica gel cartridge (InertSep Si, 100 mg, GL Sciences INC., Tokyo) preconditioned with the same solvent. The eluate was dried, dissolved in methanol (0.1 ml), and the designated test solution was used for LC/MS analysis. The clean-up procedure is presented in Fig. 1.

An Agilent 1100 LC/MSD SL system (Agilent Technologies, California) was used for LC/MS analysis. HPLC separations were performed on a Cadenza CD-C18 column (3 μm, 2 × 150 mm, Imtakt co., Kyoto, Japan) using 0.1% formic acid–methanol (15:85) as the mobile phase. The column temperature and flow rate were kept at 40 °C and 0.2 ml/min, respectively. MS parameters were set as follows: Ionization; API-ES, ion mode; positive, fragmenter voltage; 350 V, nebulizer gas; N_2 20 psi., capillary voltage; 4000 V, Dry gas; 10 l/min of N_2 at 350 °C. A 5-μl portion of the test solution was injected into the LC/MS system and positive ions of m/z 1133.5 (0–7.5 min, $[CTX1B + Na]^+$), m/z 1061.5 (7.5–15 min., $[51\text{-hydroxyCTX3C} + Na]^+$), and m/z 1045.5 (15–35 min, $[CTX3C + Na]^+$) were monitored to detect respective toxins. The synthetic standards of CTX1B, 51-hydroxyCTX3C and CTX3C used were dissolved in methanol (1 ng/ml). The signal/noise (S/N) values for the