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Toxin-screening and identification of bacteria isolated from highly toxic marine gastropod *Nassarius semiplicatus*

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ABSTRACT

Bacteria isolated from a highly toxic sample of gastropod *Nassarius semiplicatus* in Lianyungang, Jiangsu Province in July 2007, were studied to probe into the relationship between bacteria and toxicity of nassariid gastropod. The toxicity of the gastropod sample was 2×10^2 mouse unit (MU) per gram of tissue (wet weight). High concentration of tetrodotoxin (TTX) and its analogues (TTXs) were found in the digestive gland and muscle of the gastropod, using high performance liquid chromatography coupled with mass chromatography (LC-MS). Bacterial strains isolated from the digestive gland were cultured and screened for TTX with a competitive ELISA method. Tetrodotoxin was detected in a proportion of bacterial strains, but the toxin content was low. Partial 16S ribosomal DNA (rDNA) of the TTX-producing strains was then sequenced and compared with those published in the GenBank to tentatively identify the toxic strains. It was found that most of the toxic strains were closely affiliated with genus *Vibrio*, and the others were related to genus *Shewanella*, *Marinomonas*, *Tenacibaculum* and *Aeromonas*. These findings suggest that tetrodotoxin-producing bacteria might play an important role in tetrodotoxin accumulation/production in *N. semiplicatus*.

1. Introduction

Species of the caenogastropod Nassariidae have a global distribution on the soft shores and sea bed (Britton and Morton, 1994). Poisoning incidents resulted from nassariid gastropods have been reported from time to time for more than 20 years in China. From 1977 to 2000, there were 42 cases reported on shellfish poisoning in Zhoushan, Zhejiang Province due to the consumption of gastropod *Nassarius* spp. (Shui et al., 2001). In 2002, there were 50 people intoxicated, with three fatalities, in Fujian Province due to the consumption of toxic *Nassarius* spp. (Ming Pao News, 2002). More recent poisoning incidents occurred in the summer of 2004, when 55 people were intoxicated, with one fatality, in Yin Chuan, Ningxia Hui Autonomous Region, and another 15 people intoxicated in Fujian Province, due to the consumption of *Nassarius* spp. (Gao, 2004). Similar poisoning incidents caused by toxic nassariid gastropods were also reported in Taiwan (Hwang et al., 1995, 2002, 2005). Tetrodotoxin (TTX) and its analogues (TTXs) were identified as the toxic agents in nassariid gastropods corresponding for the intoxication (Hwang et al., 2002, 2005; Sui et al., 2003; Yu et al., 2007). However, in nassariid samples collected from the southern part of Taiwan, both TTX and paralytic shellfish toxins (PSTs) were detected (Hwang et al., 1995).

TTXs have been found in a wide array of gastropod species, such as trumpet shell *Charonia sauliae* (Narita et al., 1981), frog shell *Tutufa lissostoma* (Noguchi et al., 1984), Japanese ivory shell *Babylonia japonica* (Noguchi et al., 1981; Yasumoto et al., 1981), rock shells *Rapana rapiformis* and *Rapana venosa venosa* (Hwang et al., 1991a,b), lined

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moon shell *Natica lineata*, calf moon shell *Natica vitellus* and bladder moon shell *Polinices didyma* (Hwang et al., 1990, 1991b), basket shells *Niotha clathrata*, *Zeuxis scalaris* and *Zeuxis siquijorensis* (Hwang et al., 1991a,b; Narita et al., 1984), and olive shells *Oliva miniacea*, *Oliva mustelina*, and *Oliva nirasei* (Hwang et al., 2003). The origin of TTXs in those toxic gastropods remains uncovered. However, bacterium has been considered as a potential source for TTX in many TTX-bearing marine organisms (Noguchi et al., 1986; Yasumoto et al., 1986; Yotsu et al., 1987). Bacterium *Vibrio* sp., which can produce TTX and anhydrotetrodotoxin, was first isolated from the intestine of a xanthid crab, *Atergatis floridus* (Noguchi et al., 1986). After that, TTX-producing bacteria have been isolated from various marine organisms, such as *Pseudomonas* sp. from red alga *Jania* spp. (Yasumoto et al., 1986), *Pseudomonas* sp. from the skin of puffer fish *Fugu poecilonotus* (Yotsu et al., 1987), *Vibrio alginolyticus* from the starfish *Astropecten polyacanthus* (Narita et al., 1987) and the intestine of puffer fish *Fugu vermicularis vermicularis* (Noguchi et al., 1987), *Alteromonas*, *Bacillus* and *Vibrio* species from the blue-ringed octopus (*Octopus maculosus*) (Hwang et al., 1989), *Vibrio*, *Aeromonas*, *Flavobacterium* and *Pseudomonas* species from lined moon shell *Natica lineate* (Hwang et al., 1994), *Vibrio parahaemolyticus*, *V. alginolyticus*, *Pseudomonas* and *Plesiomonas* species from gastropod *N. clathrata* (Cheng et al., 1995), *V. alginolyticus* from the intestine of puffer fish *Fugu vermicularis radiatus* (Lee et al., 2000), and *Microbacterium arabinogalactanolyticum*, *Serratia marcescens* and *V. alginolyticus* from puffer fishes (Yu et al., 2004), etc. Except for marine organisms, TTX-producing bacteria have been isolated from marine and freshwater sediment (Do et al., 1990, 1991, 1993; Kogure et al., 1988). However, little information is available on potential sources of TTX in toxic nassariid gastropods in China. In this study, bacteria isolated from a highly toxic gastropod sample of *Nassarius semiplicatus* in Lianyungang, Jiangsu Province, were studied to probe into the relationship between bacteria and toxicity of nassariid gastropods.

2. Materials and methods

2.1. Sample collection

During an investigation of nassariid toxicity in Lianyungang, Jiangsu Province in 2007, highly toxic samples were found with the mouse bioassay method. About 300 individuals of *N. semiplicatus* were then collected on July 18, 2007, and transported alive to the laboratory in an ice box immediately.

2.2. Toxicity bioassay and toxin analysis of gastropod N. semiplicatus

2.2.1. Chemicals and standards

All chemicals used in toxicity bioassay and toxin analysis were analytical grade or above. Organic solvents, such as methanol and acetonitrile, were from Merck. Formic acid was from Fluka. Ultrapure water used in toxin analysis was prepared with a Millipore water purification system. TTX standard was from Calbiochem, USA.

2.2.2. Toxicity bioassay

The sample for mouse bioassay was prepared according to an AOAC method (18.086) for bioassay of PST toxicity (Williams, 1984). In our previous studies, this protocol was found a practical way for screening toxicity caused by either PSTs or TTXs, but the recovery for TTX was not examined. In brief, the whole edible portion was excised from individuals of *N. semiplicatus*, and 5 g tissue was weighed and homogenized with 10 ml 0.1 M HCl solution. The pH value was adjusted to 2.0–4.0. The mixture was heated in a boiling-water bath for 5 min, and then cooled to room temperature. The pH of the mixture was checked again and adjusted if necessary, and the final volume of the mixture was adjusted to 20 ml. The mixture was then centrifuged, and the supernatant was used for bioassay of toxicity. One milliliter supernatant was injected intraperitoneally into a mouse (strain Kunming). Based on the death time recorded, the supernatant was diluted to obtain a death time between 5 and 7 min. Three mice were injected to establish the toxicity of the sample. The toxicity was expressed in mouse units (MU), where 1 MU was defined as the amount of toxin required to kill a 20 g male mouse in 30 min (Hwang and Jeng, 1991).

2.2.3. Toxin analysis

To prepare samples for LC-MS analysis, the whole edible portion was excised from individuals of *N. semiplicatus*, a part of which was then dissected into three parts, i.e. the digestive gland, muscle and the rest part (including salivary gland, brain and mouth organs), to analyze the anatomical distribution of TTXs in different tissues. The tissues were homogenized and added with three volumes (3 ml: 1 g) of acidified 80% methanol solution (containing 1% acetic acid). After treated with a probe sonicator for 5 min, the mixture was centrifuged. The supernatant was collected, and the residue was extracted again with the same volume of methanol solution. The supernatant was combined and evaporated to dryness with a rotary evaporator. The residue was re-dissolved in 2 ml 0.05% acetic acid solution. The aqueous solution was defatted with an equal volume of dichloromethane. The upper aqueous layer was collected and filtered with an Amicon Centricon filter device (YM-3, molecular weight cut-off: 3000) previous to the analysis with LC-MS.

A Thermo Finnigan HPLC system coupled with a Finnigan LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, San Jose, CA, USA) was used to analyze TTXs. Separation of TTXs was performed on a SeQuant ZIC-HILIC silica column (150×2.1 mm, 5 μm, SeQuant AB, Sweden). Two mobile phases were used in the analysis. Mobile phase A was water, and mobile phase B was 90% acetonitrile aqueous solution. Both of the mobile phases contained 5 mM ammonium formate and 5 mM formic acid. TTXs were eluted isocratically with 30% mobile phase A at a flow rate of 100 μl/min. The injection volume was 3 μl.

The MS spectrometer for detection of TTXs was previously optimized through flow injection of TTX standard solution (1 μg/ml) at a flow rate of 5 μl/min, and the parameters were determined as followed: capillary temperature (°C) 250, ion spray voltage (kv) 4.5, sheath gas flow (arb) 25.0, capillary voltage (v) 46.0. The optimized