

Frequent occurrence of the tetrodotoxin-bearing horseshoe crab *Carcinoscorpius rotundicauda* in Vietnam

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Abstract Tetrodotoxin (TTX) was detected by HPLC and LC-MS/MS in specimens of the horseshoe crab *Carcinoscorpius rotundicauda* collected from Tan Hai village, Vung Tau province, Vietnam, where cases of poisoning due to the consumption of horseshoe crab have frequently been reported. The soft tissue from 10 out of the 12 specimens analyzed showed considerable levels of TTX, making them unsafe for human consumption (81.2 ± 50.3 MU/g). Paralytic shellfish poisoning (PSP) toxins were also detected in all of the specimens, but the levels were low. These findings show that the frequency of occurrence of *C. rotundicauda* specimens with a high level of TTX is significantly high, and that the TTX is responsible for the food poisonings caused by the consumption of *C. rotundicauda* in this area.

Keywords *Carcinoscorpius rotundicauda* · Horseshoe crab · Saxitoxin (STX) · Tetrodotoxin (TTX) · Vietnam

Introduction

In Southeast Asian countries, horseshoe crabs are often consumed as food. It has been reported that food poisonings due to the consumption of horseshoe crabs have occurred sporadically in Thailand. Two horseshoe crab species, *Tachypleus gigas* (Muller) and *Carcinoscorpius rotundicauda* (Latreille), are known to inhabit Thailand, and the latter species was responsible for all of the cases of poisoning [1–3]. Analyses of toxins in *C. rotundicauda* performed by several research groups have revealed that the species possesses paralytic shellfish poisoning (PSP) toxins and/or tetrodotoxin (TTX), indicating that these toxins caused the cases of poisoning [1–3]. These studies also revealed that the frequency of occurrence of toxic specimens was very low (3%). On the other hand, food poisonings (including fatal cases caused by the ingestion of the horseshoe crab *C. rotundicauda*) have recently occurred frequently in coastal areas of South Vietnam (Dao et al. unpublished data, 2007). In the present study, we report the result of an analysis of the toxins present in 12 specimens of *C. rotundicauda* (seven females and five males) collected from Tan Hai village, Vung Tau province, Vietnam in October 2006.

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Materials and methods

Specimens of horseshoe crab

Twelve specimens—seven females and five males—of *C. rotundicauda* (Fig. 2a) were collected from Tan Hai village, Vung Tau province, Vietnam (Fig. 1) on 26 October 2006. After transferral to the Biochemistry Laboratory at the Institute of Oceanography, Vietnam, the soft

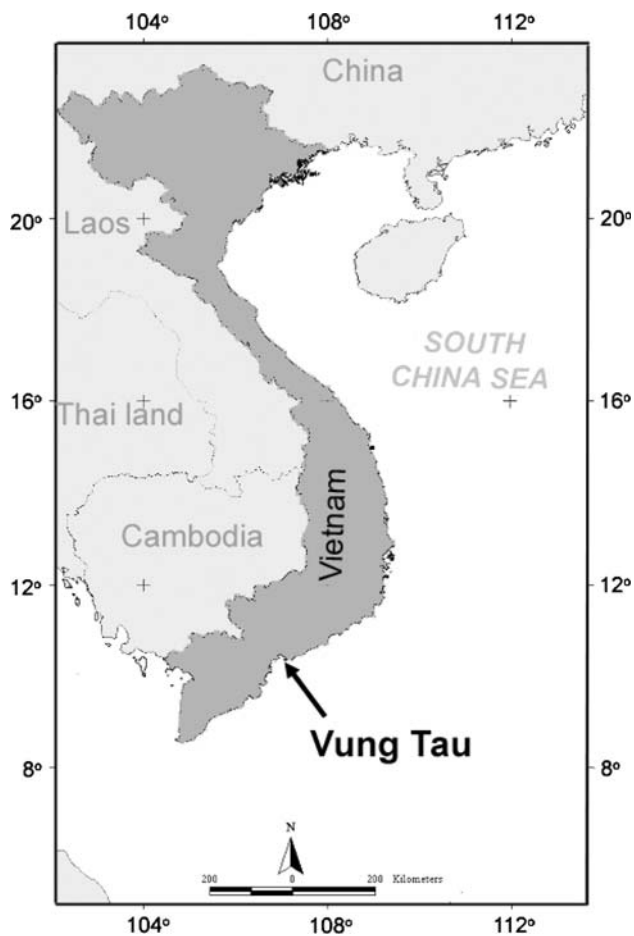


Fig. 1 Tan Hai village in Vung Tau province, where specimens of horseshoe crab *Carcinoscorpius rotundicauda* were collected

tissues were individually excised from live specimens and kept at -20°C until use.

Analysis of tetrodotoxin and paralytic shellfish poisoning toxins

The soft tissue was homogenized with an equal volume of 1% acetic acid, boiled for 5 min, and centrifuged ($8,000\times g$, 30 min). The supernatant thus obtained was used for the extract, 1 ml of which was equivalent to 0.5 g of the tissue. TTX in the extract was analyzed by HPLC according to Yotsu et al. [4]. PSP toxins were also analyzed by HPLC according to Oshima [5]. When the toxins were detected, TTX was confirmed through the application of LC-MS/MS with an ODS column (Wako Navi C18-5, 2×150 mm) using linear gradient elution from 0.2% heptafluorobutyric acid in water to 0.14% heptafluorobutyric acid in 30% acetonitrile at a flow rate of 0.2 ml/min in 10 min. Electrospray ionization (ESI) was performed in product ion scan mode, using N_2 as the desolvation, cone and collision gas (curtain gas: 50 psi; ion spray voltage:

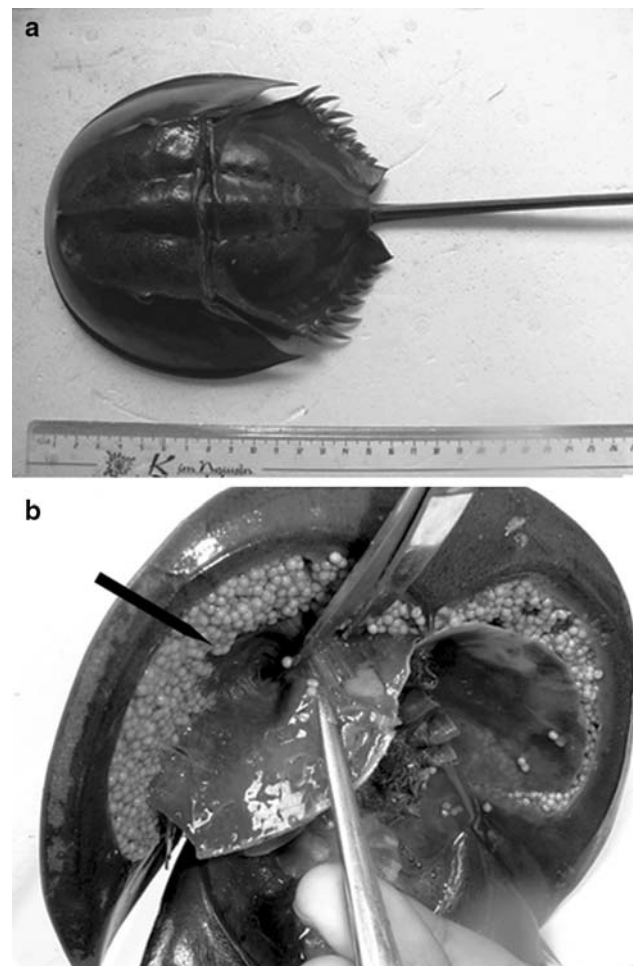


Fig. 2 Horseshoe crab *Carcinoscorpius rotundicauda*. **a** Live specimen, **b** soft tissues of a female specimen. Black arrow head indicates eggs

5,500 V; ion-source gas 1: 40 psi; ion-source gas 2: 60 psi; declustering potential: 20 V; focusing potential: 200 V; entrance potential: 10 V; collision energy: 30 eV; collision cell exit potential: 15 V). The ion at m/z 320 corresponding to $(\text{M} + \text{H})^+$ of TTX was detected in the selected ion monitoring (SIM) mode.

Results and discussion

All of the female specimens were brooding (Fig. 2b). Both the eggs and the hepatopancreas were excised together as the soft part and extracted. Figure 3 shows one of the chromatograms from the HPLC analysis of TTX. Peaks of TTX, 4-*epi*-TTX and anhydro-TTX were observed. Thus, the samples were further analyzed by LC-MS/MS to confirm the HPLC results. As shown in Fig. 4, the fragmentation pattern of the peak substance with exactly the same retention time as TTX in LC monitored at m/z 320

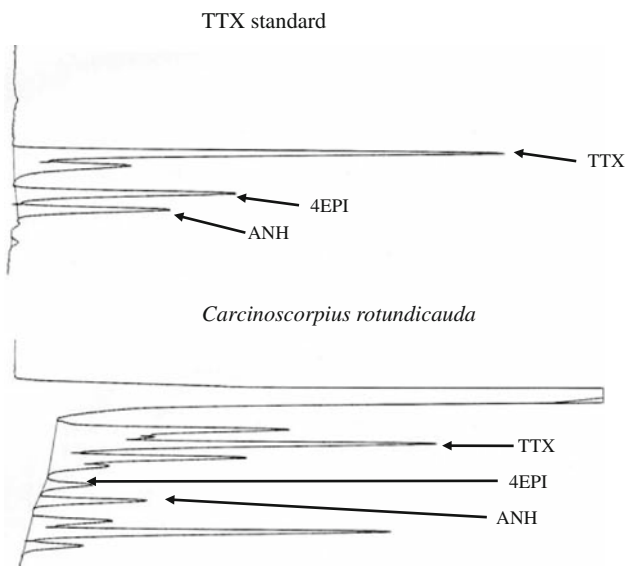


Fig. 3 HPLC chromatogram of TTX in an extract from *Carcinoscorpis rotundicauda*

in the SIM mode was indistinguishable from that of standard TTX, showing that the substance is actually TTX. Table 1 shows the toxicities of all of the specimens, as calculated based on the specific toxicity of each toxin component and expressed in mouse units (MU) [6]. One MU is the dose of toxin that will kill a 20-g male mouse (ddY) in 30 min [6]. These data show that the frequency of occurrence of toxic specimens is very high (ten out of 12 specimens; 83%). Saitanu et al. [3] analyzed more than 900 specimens of *C. rotundicauda* in Thailand, and reported that toxicity was detected in only 3% of the total specimens. Although the number of the specimens analyzed in

Table 1 TTX toxicity in *Carcinoscorpis rotundicauda*

	TTX (MU/g)	4EPI (MU/g)	ANH (MU/g)	SUM (MU/g)
f1	138.33	0.29	0.47	139.09
f2	113.18	1.40	0.90	115.48
f3	61.65	0.26	0.35	62.26
f4	101.49	0.47	0.84	102.79
f5	107.74	0.48	1.07	109.30
f6	0.00	0.00	0.90	0.90
f7	52.35	0.69	0.61	53.64
m1	17.89	0.45	0.65	18.98
m2	123.95	5.99	4.34	134.28
m3	137.15	0.13	0.23	137.50
m4	85.73	0.62	0.47	86.81
m5	0.00	9.65	4.15	13.80

MU mouse unit, f female, m male

the present study is small, the results strongly suggest that the frequency of toxic specimens of *C. rotundicauda* in Vietnam is extremely high. The results of the present study also show that the toxicity of the toxic specimens is considerable (81.2 ± 50.3 MU/g). These toxicity levels are much higher than the safe consumption level of TTX (10 MU/g) suggested in Japan [7]. On the other hand, PSP toxins were also detected in toxic specimens during HPLC analysis (Fig. 5). However, the levels of toxicity of these specimens, as calculated from specific toxicity of each component toxin [5], were close to the safety consumption level, as shown in Table 2. Thus, food poisonings due to the consumption of *C. rotundicauda* can be considered to be caused by TTX. Recently, Laymithuna et al. [8] reported the occurrence of *C. rotundicauda* containing a

Fig. 4 Confirmation of TTX in *Carcinoscorpis rotundicauda* by LC-MS/MS

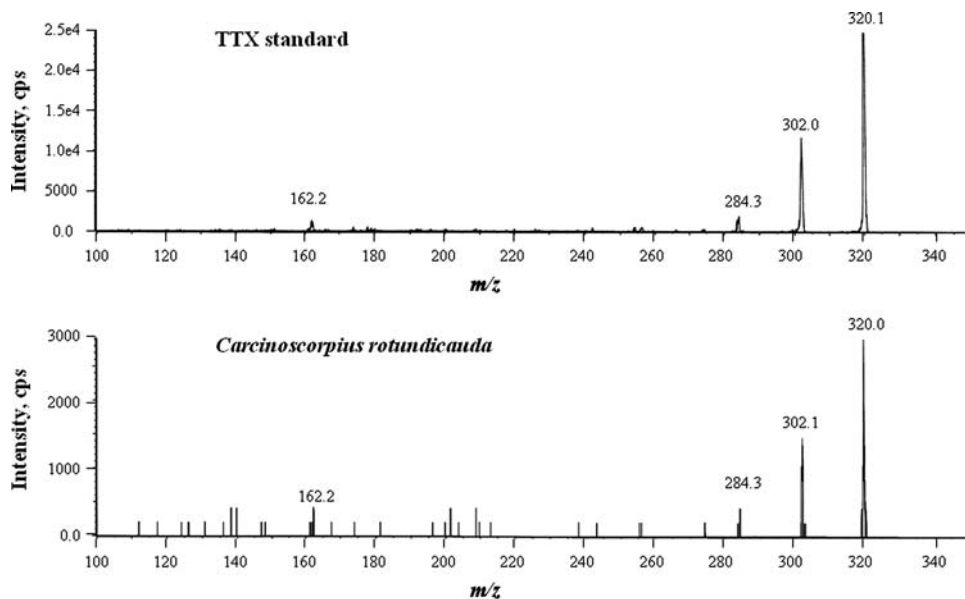


Fig. 5 HPLC chromatogram of PSP toxins in an extract from *Carcinoscorpius rotundicauda*

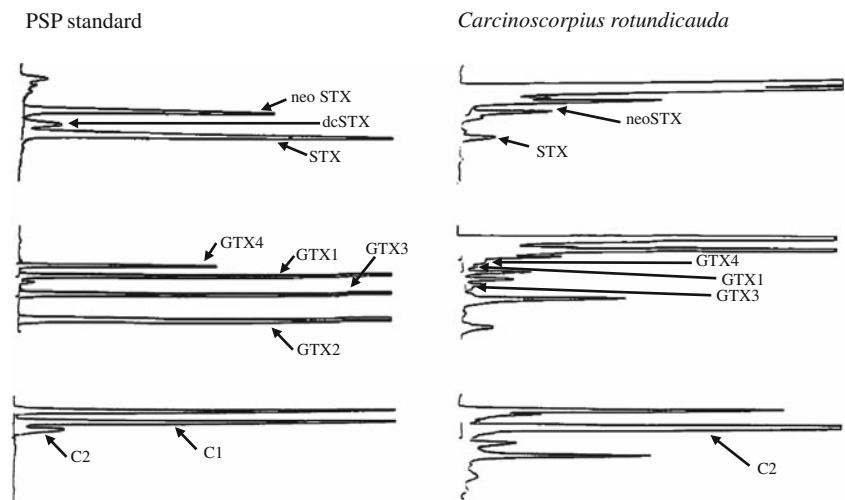


Table 2 PSP toxicity in *Carcinoscorpius rotundicauda*

	neoSTX (MU/g)	dcSTX (MU/g)	STX (MU/g)	GTX4 (MU/g)	GTX1 (MU/g)	GTX3 (MU/g)	GTX2 (MU/g)	C1 (MU/g)	C2 (MU/g)	SUM (MU/g)
f1	0.79	0.05	3.86	0.00	0.00	0.00	0.00	0.00	0.01	5.30
f2	1.35	0.00	1.07	0.00	0.00	0.00	0.00	0.00	0.09	2.52
f3	0.73	0.00	2.35	0.02	0.04	0.00	0.01	0.00	0.05	3.20
f4	0.02	0.00	3.79	0.02	0.00	0.00	0.00	0.00	0.19	4.02
f5	0.49	0.00	3.64	0.00	0.00	0.01	0.00	0.00	3.03	7.17
f6	0.19	0.05	3.69	0.04	0.18	0.00	0.00	0.00	0.18	4.32
f7	0.55	0.00	4.10	0.34	1.25	0.02	0.00	0.00	0.91	7.17
m1	0.20	0.07	3.12	0.00	0.00	0.00	0.00	0.00	1.00	4.38
m2	15.69	0.00	2.63	0.20	0.00	0.00	0.02	0.01	2.49	21.04
m3	1.16	0.02	5.04	0.09	0.00	0.00	0.00	0.00	0.60	6.93
m4	2.14	0.00	2.38	0.43	0.00	0.00	0.00	0.00	1.38	6.34
m5	0.44	0.00	5.62	0.26	0.00	0.00	0.00	0.00	0.40	6.72

MU mouse unit, *f* female, *m* male

significantly high level of TTX in Cambodia. These results, together with the present results, show that *C. rotundicauda* in South Asian countries is not suitable for human consumption.

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