TETRODOTOXIN POISONING

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Tetrodotoxin (TTX) is one of the most potent and oldest known neurotoxins. The poisoning cases due to ingestion of TTX-containing marine animals, especially for puffer, have frequently occurred in Asia since a long time ago. This chapter describes various topics on TTX poisoning including the tendency of poisoning incidents, typical case report, treatment and prevention, biology distribution, original source, infestation mechanism, detection methods, characteristics of chemistry and pharmacology, and therapeutic application.

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Furthermore, the protocols for how to make puffer safe to eat and how to prevent puffer products made from toxic puffers have been suggested. Finally, the biological significance and neurophysiological role of TTX have been elucidated and TTX may act as an important drug like anesthetic in future.

I. INTRODUCTION

Tetrodotoxin (TTX), a puffer toxin named after its order name Tetraodontiformes by Professor Y. Tahara in 1880, is one of the most potent nonproteinaceous toxins known, responsible for numerous fish poisonings. This toxin is one of the oldest known natural toxins recorded as early as 2700 BC in Chinese literature, describing the toxicity of puffer, and around 2500 BC in Egyptian history.

TTX poisoning cases due to ingestion of puffer have frequently occurred especially in Japan where these fish have been a traditional food since a long time ago. The preferred forms of this delicacy are slices of raw flesh ("sashimi") and slightly cooked liver ("kimo"), Japanese people are aware of the puffer's toxicity and have devised ways to reduce TTX in the liver. However, TTX poisoning incidents continue to occur in Japan yet. Since there is no cure for the poisoning patient, its mortality is very high. Judging from the statistics (Table I) provided by Japanese Ministry of Health and Welfare (MHW), the number of deaths due to puffer poisoning have steadily declined, from more than 10 cases with high mortality every year between 1960 and 1981 to less than 10 cases with low mortality every year since 1982 (MHW, 1997; Noguchi, 2003). This decline in incidence is probably not only due to complete performance of government regulations but also due to increase of cultured puffer (nontoxic) instead of decrease of wild puffer (toxic).

TTX is a heterocyclic guanide compound whose chemical structure has been characterized (Figure 1). TTX has one guanidium moiety. As being seen in Figure 2, many derivatives of TTX have been found, although their toxicities vary widely. Since being first isolated in 1964 from puffer of the family Tetraodontidae (Tsuda *et al.*, 1964), TTX has been found in diverse organisms, including marine goby and an octopus as well as some terrestrial amphibians such as newts and frogs (Table II). TTX has been found in several bacterial species, including *Shewanella* sp. and *Vibrio* spp., and is now believed to be of bacterial origin.

Recently, several poisoning cases due to ingestion of, in addition to those of puffers, big and small gastropods have caused in Japan, Taiwan, and China. Especially small gastropods in China have caused many poisoning for a long time, resulting in many deaths and caused serious problems in public health.

TABLE I
PUFFER POISONING INCIDENTS IN JAPAN[a]

Year	Number of incidents	Number of patients	Number of deaths	Mortality (%)
1965	106	152	88	57.9
1970	46	73	33	45.2
1975	52	75	30	40.0
1980	46	90	15	16.7
1981	30	46	12	26.0
1982	26	33	8	24.2
1983	18	34	6	17.6
1984	23	39	6	15.4
1985	30	41	9	21.9
1986	22	38	6	15.8
1987	35	52	4	7.7
1988	26	46	5	10.9
1989	31	45	5	11.1
1990	33	55	1	1.8
1991	29	45	3	6.7
1992	33	57	4	7.0
1993	28	44	4	9.1
1994	16	23	1	4.3
1995	30	42	2	4.8
1996	21	34	3	8.8
1997	28	44	6	13.6
1998	27	39	4	10.3
1999	20	34	2	5.9
2000	29	40	0	0
2001	31	52	3	5.8
2002	32	49	5	10.2

[a]Data cited from MHW (1997) and Noguchi (2003).

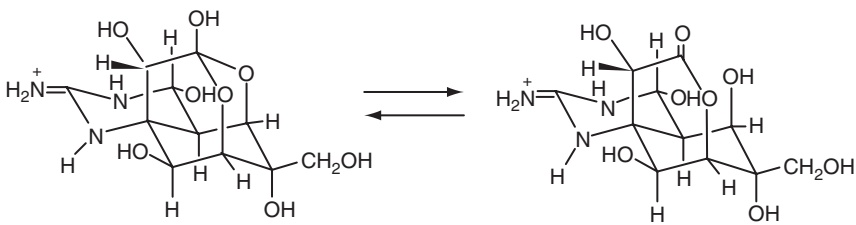
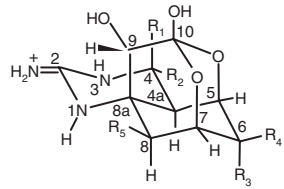


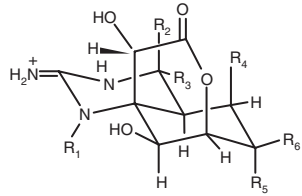
FIG. 1 The tautomers of TTX.

(1) Hemilactal type



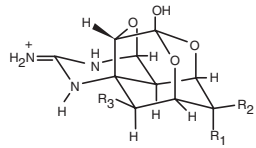
	R_1	R_2	R_3	R_4	R_5
TTX	H	OH	OH	CH_2OH	OH
4-*epi*TTX	OH	H	OH	CH_2OH	OH
6-*epi*TTX	H	OH	CH_2OH	OH	OH
11-*deoxy*TTX	H	OH	OH	CH_3	OH
6-*epi*-11-*deoxy*TTX	OH	H	OH	CH_3	OH
TTX-8-*O*-hemisuccinate	H	OH	OH	CH_2OH	$OOC(CH_2)_2COO^-$
Chiriquitoxin	H	OH	OH	*R* *S* $CH(OH)CH(NH_3^+)COO^-$	OH
11-*nor*TTX-6(*S*)-ol	H	OH	OH	H	OH
11-*nor*TTX-6(*R*)-ol	H	OH	H	OH	OH
11-*nor*TTX-6,6-diol	H	OH	OH	OH	OH
11-*oxo*TTX	H	OH	OH	$CH(OH)_2$	OH
TTX-11-carboxylic acid	H	OH	OH	COO^-	OH

(2) Lacton type

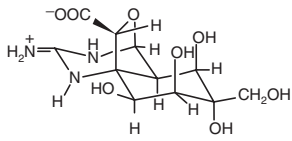


	R_1	R_2	R_3	R_4	R_5	R_6
TTX (lactone)	H	H	OH	OH	OH	CH_2OH
6-*epi*TTX (lactone)	H	H	OH	OH	CH_2OH	OH
11-*deoxy*TTX (lactone)	H	H	OH	OH	OH	CH_3
11-*nor*TTX-6(*S*)-ol (lactone)	H	H	OH	OH	OH	H
11-*nor*TTX-6(*R*)-ol (lactone)	H	H	OH	OH	H	OH
11-*nor*TTX-6,6-diol (lactone)	H	H	OH	OH	OH	OH
5-*deoxy*TTX	H	H	OH	H	OH	CH_2OH
5,11-*dideoxy*TTX	H	H	OH	H	OH	CH_3
6-*epi*-5,11-*dideoxy*TTX	H	OH	H	H	OH	CH_3
1-hydroxy-5,11-*dideoxy*TTX	OH	H	OH	H	OH	CH_3
5,6,11-*trideoxy*TTX	H	H	OH	H	H	CH_3
4-*epi*-5,6,11-*trideoxy*TTX	H	OH	H	H	H	CH_3

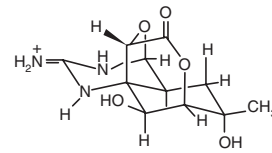
(3) 4,9-Anhydro type



	R_1	R_2	R_3
4,9-Anhydro TTX	OH	CH_2OH	OH
4,9-Anhydro-6-*epi*-TTX	CH_2OH	OH	OH
4,9-Anhydro-11-*deoxy*TTX	OH	CH_3	OH
4,9-Anhydro-TTX-8-*O*-*hemisuccinate*	OH	CH_2OH	$OOC(CH_2)_2COO^-$
4,9-Anhydro-TTX-11-*O*-*hemisuccinate*	OH	$CH_2OCO(CH_2)_2COO^-$	OH



Tetrodonic acid (tetrodoic acid)



4,9-Anhydro-5,11-*dideoxy*TTX

FIG. 2 The structure of three types of TTX analogues. (1) Hemilactal type, (2) Lacton type, and (3) 4,9-Anhydro type (Yotsu-Yamashita, 2001).

Accordingly, TTX in TTX-bearing organisms is found to come from concentration through several steps of the food web, starting with TTX production by bacteria.

II. TTX POISONING

A. INCIDENTS IN THE WORLD

1. Japan

In Japan, many puffer poisoning cases occur every year, resulting in numerous deaths with approximately 8.3% mortality over the past 10 years (Table I). Japanese people know that puffer, especially its liver (“kimo”), is very toxic, but more than a few “kimo” fans dare to ingest the liver, believing that the toxin has been eliminated from it using their own “special” (traditional) detoxification method. Consequently most puffer poisoning cases are caused by ingestion of toxic livers. Since the Japanese MHW published a guideline of edible puffers in 1983, with updates in 1993 and 1995 (Table III) (MHW, 1983), these guidelines only prohibit puffer livers to be served in restaurants. As a result, many TTX poisoning still occur due to

TABLE II
DISTRIBUTION OF TTX IN ANIMALS

	Animals		Part
1	Platyhelminthes	Flatworms	
	Turbellaria	*Planocera* spp.	Whole body
2	Nemertinea	Ribbon worms	Whole body
		Lineus fuscoviridis	Whole body
		Tubulanus punctatus	Whole body
		Cerebratulus lacteus	Whole body
		Cephalothrix linearis	Whole body
3	Mollusca	*Charonia sauliae*	Digestive gland
	Gastropoda	*Babylonia japonica*	Digestive gland
		Tutufa lissostoma	Digestive gland
		Zeuxis siquijorensis	Digestive gland
		Niotha clathrata	Digestive gland
		Natica lineata	Whole body
		Rapana spp.	Digestive gland
		Cymatium echo	Digestive gland
		Pugilina ternotona	Digestive gland
	Cephalopoda	*Hapalochlaena maculosa*	Postsalivary gland
4	Annelida	*Pseudopotamilla occelata*	Whole body
	Polychaeta	*Lepidonotus helotypus*	Whole body
		Halosydna brevisetosa	Whole body
		Harmothoe imbricata	Whole body
5	Arthropoda	*Atergatis floridus*	Whole body
		Zosimus aeneus	Whole body
		Carcinoscorpius rotundicauda	Egg
6	Chaetognatha	Arrowworms	
		Eukrohonia hamata	Head
		Parasagitta spp.	Head
		Flaccisagitta spp.	Head
7	Echinodermata	Starfish	
		Astropecten polyacanthus	Whole body
		Astropecten latespinosus	Whole body
		Astropecten scoparius	Whole body
8	Vertebrate	*Takifugu* spp.	Skin, liver, ovary
	Pisces	*Yongeichthys criniger*	Skin, viscera, gonad
	Amphibia	*Taricha* spp.	Skin, egg, ovary, muscle, blood
		Notophthalmus spp.	Skin, egg, ovary
		Cynops spp.	Skin, egg, ovary, muscle, blood
		Triturus spp.	Skin, egg, ovary, muscle, blood
		Ambystoma sp.	Skin, egg, ovary, muscle
		Paramesotriton sp.	Skin, egg, ovary, muscle
		Polypedates sp.	Skin
		Atelopus spp.	Skin
		Colostethus spp.	Skin
9	Red Clacareous alga	*Jania* spp.	Whole body
10	Dinoflagellate	*Alexandrium tamarense*	Whole body

TABLE III
EDIBLE PART OF PUFFER IN JAPAN

Family	Species	Edible part		
		Muscle	Skin	Male gonad
	"Kusafugu" *Takifugu niphobles*	○	—	—
	"Komonfugu" *Takifugu poecilonotus*	○	—	—
	"Higanfugu" *Takifugu pardalis*	○	—	—
	"Shousaifugu" *Takifugu snyderi*	○	—	○
	"Nashifugu" *Takifugu vermicularis*[a]	○	—	○
	"Mafugu" *Takifugu porphyreus*	○	—	○
	"Mefugu" *Takifugu obscurus*	○	—	○
Tetraodontidae	"Akamefugu" *Takifugu chrysops*	○	—	○
	"Torafugu" *Takifugu rubripes*	○	○	○
	"Karasu" *Takifugu chinensis*	○	○	○
	"Shimafugu" *Takifugu xanthopterus*	○	○	○
	"Gomafugu" *Takifugu stictonotus*	○	—	○
	"Kanafugu" *Lagocephalus inermis*	○	○	○
	"Shirosabafugu" *Lagocephalus wheeleri*	○	○	○
	"Kurosabafugu" *Lagocephalus gloveri*	○	○	○
	"Yoritofugu" *Sphoeroides pachygaster*	○	○	○
	"Sansaifugu" *Takifugu flavidus*	○	—	—
	"Ishigakifugu" *Chilomycterus reticulatus*	○	○	○
Diodontidae	"Harisenbon" *Diodon holocanthus*	○	○	○
	"Hitozuraharisenbon" *Diodon liturosus*	○	○	○
	"Nezurnifugu" *Diodon hystrix*	○	○	○
Ostracidae	"Hakofugu" *Ostraction immaculatum*	○	—	—

[a]Applicable to muscle of the species caught in Shimabara Bay, Tachibana Bay, and the Inland Sea of Kagawa and Okayama, and to male gonad in Shimabara Bay and Tachibana Bay.
○, Edible; —, nonedible.

consumption of homemade puffer liver dishes from wild fish usually caught by a family member.

Recently, 80% of puffer in fish market are cultured and rarely are they toxic. Accordingly, puffer is imaged from toxic to nontoxic. On the contrary, the poisoning events due to ingestion of other marine animals bearing TTX have occurred. The toxicity of puffers is related to their spawning with the highest toxicity levels, especially in the ovary, between March and June in Japan.

The two typical examples of TTX poisoning cases in Japan are as follows:

1. Poisoning due to the liver of the finepatterned puffer (*Takifugu poecilonotus*)

A 48-year-old man in Nagasaki City, Nagasaki Prefecture, ate more than four slices of slightly cooked liver ("kimo") along with some flesh of wild

T. poecilonotus in the evening in October 1996. The fish had been caught earlier in the day. After 30–60 minutes of ingestion of the puffer tissues, the man began to suffer from numbness in his hands and limbs, followed by cyanosis and respiratory failure during the next 60 minutes. Although he was immediately hospitalized, the patient died during the following hour.

TTX toxicity of puffer is commonly determined by mouse bioassay. The mouse bioassay is performed by intraperitoneal injection. After injection with TTX-associated extracts, the mice show the characteristic signs and symptoms, like unique scratching of the shoulders/mouth by their hind limbs, weakness progressing to paralysis in hind limbs, uncoordinated movement, shallowness of breathing, convulsion, and jumping, followed by respiratory failure. Toxicity of an extract is expressed in terms of mouse units (MU), where 1 MU is defined as the amount of TTX required to kill a 20-g male of ddY or ICR strain in 30 minutes (Hwang and Jeng, 1991; MHW, 1991). Minimum detectable limit is about 0.2 μg of TTX (1 MU) per assay. In these cases, toxicity scores of the puffer livers and flesh were 715–4260 MU/g (equivalent to 0.14–0.85 mg of TTX per gram) and less than 5 MU/g (equivalent to 0.001 mg of TTX per gram), respectively. The cause of death was determined to be TTX in the wild *T. poecilonotus* liver. The victim died due to ingestion of a total toxicity score of more than 10,000 MU (about 2-mg TTX), equivalent to the LD_{50} of TTX for man (Noguchi and Akaeda, 1998).

2. Poisoning due to the digestive gland of a big gastropod trumpet shell (*Charonia sauliae*)

In December 1979, a 41-year-old man ate a boiled digestive gland (~60 g) of "boshubora" or trumpet shell (*C. sauliae*) that he had caught in the Shimizu Sea. Thirty minutes after ingestion, he was immediately hospitalized and received artificial respiration. Although he remained unconscious for 2 days, he recovered fully in 5 days.

The leftover digestive gland of the *C. sauliae* showed a toxicity score of 17,000 MU (equivalent to 3.4 mg of TTX) in the bioassay for TTX (Narita *et al.*, 1981). Thus, the victim may have ingested about 10,000 MU (2 mg of TTX), the approximate LD_{50} of the toxin for man. TTX was also identified as the causative agent in samples from similar TTX poisoning cases in Wakayama in 1982 and in Miyazaki in 1987 using thin-layer chromatography (TLC), electrophoresis, and instrumental analyses with infrared (IR), proton nuclear magnetic resonance (^{1}H NMR), and gas chromatography–mass spectrometry (GC–MS).

2. Taiwan and China

In Taiwan and China, many food poisoning cases due to ingestion of wild puffer have occurred (Hwang, 2003a,b), while consumers here do not often eat puffer. According to data of TTX poisoning in Taiwan (Table IV), TTX

TABLE IV
FOOD-BORNE OUTBREAKS CAUSED BY TTX IN TAIWAN, 1994–2003

Time		Food	Patients/deaths	Place
1994	January, April, May	Puffer	10/0	Changhua, Taitung, Pingtung
	April, May	Gastropod	26/0	Pingtung
1995	June	Goby	2/0	Miaoli
1997	February, December	Puffer	4/1	Changhua, Kaohsiung
1997	February, March	Goby	5/0	Kaohsiung, Tainan
1998	May, June, December	Dried dressed fish fillet	5/0	Taitung, Tuaoyuan, Taipei
1999	January	Puffer	18/1	Taipei, Changhua
2000	January, March, August	Puffer	14/0	Changhua, Keelung
	February, April	Pried dressed fish fillet	7/2	Changhua, Tainan
	July	Gastropod	1/0	Chaiyi
2001	March, April	Puffer	13/1	Taichung, Keelung, Taipei, Lunlin
	April	Flake of dried mullet roe	1/0	Kaohsiung
	April	Gastropod	5/0	Taipei
2002	February	Gastropod	1/0	Pingtung
	March	Puffer	6/3	Taitung
2003	March, September	Puffer	6/0	Kaohsiung

Total: 30 outbreaks, 124 patients, 8 deaths.

Source: Hwang *et al.* (2003).

poisoning cases seem to occur by ingesting puffer roe as fake of "karasumi" dried mullet roe, toxic puffer muscle by mistake, and the dried dressed fish fillet produced from toxic puffers (Du *et al.*, 1999; Hsieh *et al.*, 2003; Hwang *et al.*, 2002a). New faces of small gastropods as causative foods of TTX poisoning newly appear, such as *Niotha clathrata, Zeuxis scalaris, Natica vitellus, Oliva miniacea, Oliva mustelina, Oliva hirasei*, and *Nassarius glans* (Hwang *et al.*, 1995, 2002b, 2003, 2005; Shiu *et al.*, 2003). In China, food poisoning cases due to ingestion of puffer have occurred. Recently, the epidemic investigation on small gastropod poisoning incidents in Zhoushan has been shown in Figure 3. The toxic organism and causal agent were identified as *Zeuxis samiplicutus* and TTX (Shui *et al.*, 2003; Sui *et al.*, 2002).

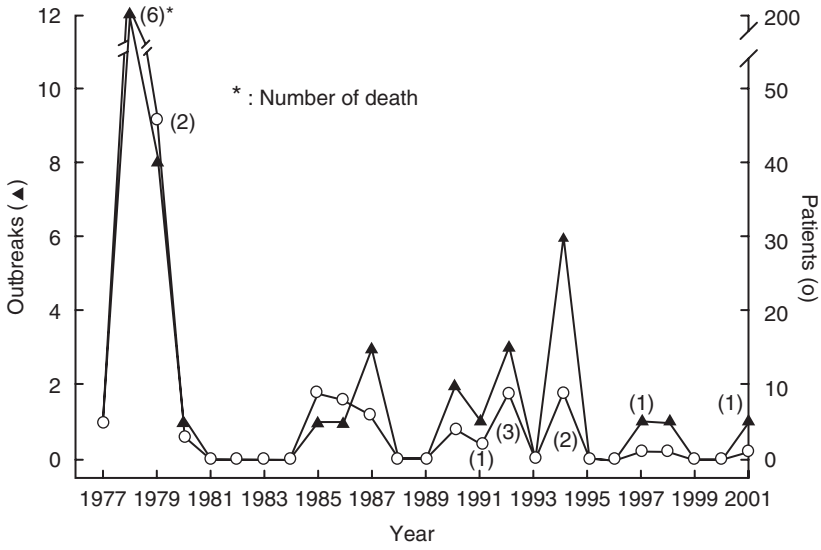


FIG. 3 Yearly distribution of snail poisoning outbreaks and patients in Zhoushan City (Shui *et al.*, 2003).

The two typical examples of TTX poisoning cases in Taiwan are as follows:

1. A food poisoning incident due to ingestion of an unknown fish

A food poisoning incident following fish ingestion occurred in Chunghua Prefecture, western Taiwan on January 2, 2000. A total of five victims (four men, 58- to 64-year old, and one woman, 46-year old) were reported. Symptoms included paralysis, coma, nausea, vomiting, ataxia, aphasia, and difficulty of respiration. Among these victims, two men suffered from more serious symptoms and were treated with intravenous fluids, mechanical ventilation, and intensive treatment in the hospital. They were then discharged uneventfully after 1 week of management. Presently, all patients are normal and healthy. The fish, which might be puffer was reported by victims, was collected from the coastal area of Chunghua Prefecture. The small residue (11.02 g) of cooked fish liver was retained by the victims in this incident.

The cooked fish liver retained by the victims was assayed for toxicity and mitochondrial DNA. Meanwhile, eight live specimens of puffer *Takifugu niphobles* were also assayed. The toxicity of cooked fish liver was 280 MU/g. All specimens of *T. niphobles* showed high toxicity (more than 850 MU/g) in the liver. The toxin from cooked fish liver and liver of *T. niphobles* was identified to be TTX. The cooked fish and fresh liver of *T. niphobles* showed the same

sequence genotype and the same single restriction site for *Bsa*I. Therefore, the species of cooked fish liver was suggested as *T. niphobles* (Hsieh *et al.*, 2002).

2. Goby intoxication in a uremic patient

The patient was a 52-year-old uremic woman. The uremia was of undefined etiology. Over the past 3 years she has received regular hemodialysis. One day in April, 1998, she and her husband, a healthy 55-year-old man, had fish soup. About 4 hours after the meal she developed a headache and a lingual and circumoral tingling sensation and numbness at the distal parts of all four limbs. She was dizzy and unsteady, had difficulty in swallowing, and became very weak. She was taken to the emergency service and was placed on machine-assisted ventilation as respiratory distress and cyanosis developed. Her husband remained asymptomatic throughout this time.

The patient's condition kept on deteriorating, developing eventually into a comatous-like state with no spontaneous or reflexive eye or limb movement within 30 minutes of intubation. On neurological examination, the papillary light reflex was absent and oculocephalic maneuver elicited no ocular movements. All four limbs were areflexic and Bafinski's signs were absent. Brain computerized tomography (CT) and laboratory studies of arterial blood gas (under assisted ventilation), electrolytes, liver function, blood glucose, and cerebrospinal fluid (CSF) were unremarkable. An examination of renal function indicated chronic renal insufficiency with mild azotemia. An electric encephalography (EEG) recorded 18 hours after the onset of symptoms when the neurological condition was unchanged, showed posterior dominant alpha waves intermixing with trains of short duration, diffuse theta waves. At the same time brief noxious stimuli were replaced transiently by beta activities. The findings suggested that the profound neurological dysfunction might be peripheral in origin. The patient was given a course of hemodialysis according to the set schedule for uremia at 21 hours after onset of the symptoms. Her condition improved dramatically within an hour. She could open her eyes and she communicated and answered questions correctly by blinking. Pupillary reflex recovered and voluntary eye movements were limited only at the extreme lateral gaze. Muscle power was grade 3 and 4 in the proximal and distal parts of the four limbs. Tendon reflexed were still absent. She was taken off mechanical ventilation the next day. Her clinical condition continued to improve and her symptoms subsided in a stepwise pattern, in response to each course of hemodialysis. She regained her initial strength by the time she was discharged on day 16.

When analyzing the remains of the cooked fish (identified as *Yongeichthys nebulosus*), TTX was demonstrated by TLC, high-performance liquid chromatography (HPLC), and cellulose acetate membrane electrophoresis. Toxicity was assayed by using ICR (Institute of Cancer Research) strain adult male mice and the toxicity score was 25 MU/g in fish muscle. The synergistic effect of uremia and TTX is obvious in this incident in which the patient and her

husband ingested roughly an equal amount of TTX (about 0.2 mg, calculated from toxic score times the weight of ingested fish). The amount is about 10% of the estimated lethal dose in humans and caused no clinical evidence of poisoning in the healthy person. In this case, hemodialysis is suggested as an effective method in the treatment of TTX intoxication (Lan *et al.*, 1999).

3. Other countries

Two Dutch sailors died within 17–20 minutes after ingestion of the liver of a South African puffer (Halstead, 1987; Noguchi and Ebesu, 2001). Ten human fatalities from the consumption of puffer have been reported in the United States (Ebesu *et al.*, 2000). Three of them occurred between 1908 and 1990 (Lange, 1990; Redy and Hayes, 1989) and four in Hawaii between 1903 and 1925, all of the poisoning originated from the consumption of *Arothron* sp. (Helfrich, 1963). In 1986, a further poisoning incident occurred in Hawaii due to consumption of the liver of *Diodon hystrix*, affecting one man (Sims and Ostman, 1986). Although the victim did not attend hospital until 24 hours of exposure, he recovered within 1 week.

In Bangladesh on November 16, 1998, a food poisoning incident due to ingestion of roe of a puffer *Takifugu oblongus* occurred, affecting eight people inclusive of five deaths (Mahmud *et al.*, 1999b). Their symptoms were as follows: dyspnea, numbness of the lips, paralysis, and stomachache followed by vomiting. These appeared after 2 hours of ingestion. Two victims became unconscious within 5 hours of exposure. On the way to the hospital, two among eight patients died while the remaining six were admitted. Three of the six patients died in the hospital and three recovered and left the hospital.

Poisonings due to ingestion of horseshoe crab (*Carcinoscorpius rotundicauda*) eggs have occasionally been reported in Thailand (Smith, 1933; Tiammeth, 1953; Trishnananda *et al.*, 1966). The symptoms of the victims were mostly similar to these caused by TTX or paralytic shellfish poisons (PSPs). The responsible toxin was identified as PSP as the major toxin, with a minor unknown toxin (Fusetani *et al.*, 1982, 1983). *C. rotundicauda* toxin was known to consist of mainly TTX, anhydroTTX, and only a small amount of saxitoxin (STX) and neosaxitoxin (neoSTX) (Kungsuwan *et al.*, 1987).

B. SYMPTOMS AND SIGNS

In human, the MLD_{50} of TTX is approximately estimated to be 10,000 MU, equivalent to 2 mg of TTX. Minimum dose for developing TTX poisoning symptoms in human is supposed to be near MLD_{50}. TTX is heat-resistant and in general cooking process it is not decomposed.

TABLE V
THE MAIN DEGREE OF TETRODOTOXICATION AND ASSOCIATED SYMPTOMS

Degree	Characteristic symptoms
First	Neuromuscular symptoms (paresthesia of lips, tongue, and pharynx; taste disturbance; dizziness; headache; diaphoresis; pupillary constriction); gastrointestinal symptoms (salivation, hypersalivation, nausea, vomiting, hyperemesis, hematemesis, hypermotility, diarrhea, abdominal pain)
Second	Additional neuromuscular symptoms (advanced general paresthesia; paralysis of phalanges and extremities; pupillary dilatation, reflex changes)
Third	Increased neuromuscular symptoms (dysarthria; dysphagia, aphagia; lethargy; incoordination, ataxia; floating sensation; cranial nerve palsies; muscular fasciculations); cardiovascular/pulmonary symptoms (hypotension or hypertension; vasomotor blockade; cardiac arrhythmias including sinus bradycardia, asystole, tachycardia, and atrioventricular node conduction abnormalities; cyanosis; pallor; dyspnea); dermatologic symptoms (exfoliative dermatitis, petechiae, blistering)
Fourth	Respiratory failure, impaired mental faculties, extreme hypotension, seizures, loss of deep tendon and spinal reflexes

Tetrodotoxication is characterized by a few symptoms of the victims. The type, severity, and range of symptoms depend on the amount of toxin ingested, age, and health of the victim. The four main stages or degrees of tetrodotoxication based are shown in Table V.

First degree is as follows:

Depending on the amount of toxin ingested, symptoms usually appear within 10–45 minutes of exposure, though some cases have been reported to be a symptomatic until as much as 3–6 hours after exposure. Oral paresthesia is usually the initial symptom and gradually spreads to the extremities and trunk. Other early symptoms include taste disturbance, dizziness, headache, diaphoresis, and papillary symptoms of salivation, hypersalivation, nausea, vomiting, hyperemesis, hematemesis, hypermotility, diarrhea, and abdominal pain. These symptoms are characteristic of first degree of tetrodotoxication in a system devised by Fukuda and Tani (1941).

Second degree is as follows:

Symptoms are characterized by additional neuromuscular ones, such as advanced general paresthesia, paralysis of phalanges and extremities, papillary dilation, and loss of papillary and corneal reflexes. Respiratory distress may also begin.

Third degree is as follows:

Patient's experience increased neuromuscular symptoms. Paresthesia of the larynx may lead to dysphagia and aphagia. Other neuromuscular symptoms include dysarthria, lethargy, muscular incoordination, and ataxia: sensations of

floating due to numbness; cranial nerve palsies; and muscular fasciculations. Cardiovascular and pulmonary symptoms of hypotension or, more rarely, hypertension; vasomotor blockade; cardiac arrhythmias including sinus bradycardia, asystole, tachycardia, and atrioventricular node conduction abnormalities; cyanosis, pallor, and dyspnea may also occur during this stage of intoxication. Dermatologic symptoms of exfoliative dermatitis, petechiac, and blistering are also often observed.

Fourth degree is as follows:

Symptoms involve respiratory failure, extreme hypotension, seizures, and loss of deep tendon and spinal reflexes. Although some patients may exhibit impaired mental faculties and may even become comatose, most patients remain fully conscious in 6–24 hours, if the patient survives past 24 hours the prognosis for recovery is good. Otherwise, death is caused by progressive ascending paralysis involving the respiratory muscles.

In addition to the symptoms just described, several unusual symptoms have been reported in a few cases. These include hypertension (Deng *et al.*, 1991; Yang, 1967) and cranial diabetes insipidus (Tambyah *et al.*, 1994). In the patients who experienced dramatic increases in blood pressure in response to TTX, Deng *et al.* (1991) noted they all had preexisting hypertension and sensitivity to sympathetic stimulation. The case involving cranial diabetes insipidus is described below.

C. TREATMENT

Although monoclonal anti-TTX antibody to detect TTX has recently been developed, there are no known antidotes or antitoxins to TTX.

So treatment of symptoms is supportive. Diagnosis is based on the clinical symptoms and history of consumption of toxic organisms. To reduce exposure to unabsorbed TTX, emetics may be administered if vomiting has not already occurred. In addition, gastric lavage, especially with 2% sodium bicarbonate, followed by activated charcoal is recommended (Sims and Ostman, 1986). Fluid and electrolyte replacement therapy may be used to reduce resulting fluid loss. Atropine may also be given to counteract hypotension and bradycardia (Sims and Ostman, 1986). In cases of respiratory difficulty or failure, oxygen and other ventilatory support, including endotracheal intubation, is often necessary. Lan *et al.* (1999) suggested that hemodialysis might be an effective method in the treatments of TTX intoxication.

Several researchers report that administration of the anticholinesterases edrophonium and neostigmine enhance the recovery of motor power and markedly reduce paresthesia and numbness (Chew *et al.*, 1983, 1984; Sorokin, 1973; Tan, 1980; Torda *et al.*, 1973). Although these reports are contrary to those of Kao (1966), Chew *et al.* (1983, 1984) suggest that TTX causes a

competitive reversible block at the motor end-plate as well as at the motor axon and muscle membrane. The effectiveness of the anticholinesterases can thus be explained by their action of increasing the quantal release of acetylcholine at the neuromuscular junction, thereby reversing the TTX blockage (Chew *et al.*, 1983).

D. PREVENTION

Puffer poisoning occurs when people always intentionally consume toxic specimen or its tissues. In many cases, the consumers cannot differentiate the toxic puffer species, the strong toxic parts of puffer, and other TTX-bearing animals (toxic gobies, gastropods, crabs, horseshoe crabs, and so on). Hence, TTX-associated poisoning incidents do not minimize or prevent.

In Japan, puffer fish is eaten primarily between October and March. Production of the traditional "fugu-kimo" is in the speciality stores of "puffer fish." Livers separated from the whole body of wild puffer fish are used to squeeze TTX by repeatedly many presses with both hands after being run with a lot of water over night and then boiled with dilute saline solution, resulting in being served as "dish of puffer fish liver" with special vinegar. However, a little bit of TTX is generally left in the prepared liver, which is no problem to cause a food poisoning. Food poisonings due to ingestion of the liver generally do not occur in the speciality stores of "puffer fish" since according to the secret manual handed down there for a long time to eliminate TTX, the liver had been treated. Most of puffer fish poisonings occur in the family. Many patients die due to their incomplete treatment to release TTX from the toxic livers. A practical TTX elimination method is dependent on each speciality store of "puffer fish." The method is secret, never opened.

To prevent the gourmets from ingestion, it is the best way to advise them not to take the liver of toxic puffer. Recently, because harvest of wild puffer is decreasing, cultured puffer is flourishingly reared in many districts of Japan, which is supplied to meet up the big demand of consumer. Hopefully, these puffer fish reared in net in Japan are found to be nontoxic. In near future, nontoxic liver of these puffers generally may be served to the gourmets, with safety.

On the other hand, to ensure the safety of consumers in some other Asian countries where the puffer consumption has been increasing, a comprehensive toxicological study should be carried out to identify the toxic and nontoxic puffer fish. On the basis of the results, consumer awareness should be created through several media in order to reduce the poisoning cases. In Japan, as described before, the death number decreases to less than one order although food poisoning cases occur every year.

In Taiwan, puffer food poisoning occasionally occur due to mistaking toxic species and dried dressed fish fillet produced from toxic puffer fish

(Lin *et al*., 2002a). Hwang *et al*. (1994a) indicated that the toxic puffer fillet can be treated with 3% NaCl to salt out more than 80% toxin at 4°C for 24 hours. The processed fillets become nontoxic and can be used as the material of dried dressed fish fillet. Another possible way to ultilize toxic puffer meat is first to avoid the toxin contamination from liver and ovary. Then the puffer meat is washed by 0.3% NaCl water, mixed with other nontoxic fish meat to form minced fish meat, and then produced into minced products such as fish ball, fish sausage, ''kamaboko,'' ''chikuwa,'' and ''tempura.'' This process includes the salting out to eliminate the toxin from puffer meat and mixing other nontoxic fish meat to minimize the toxin in the final products. However, any final puffer products should not contain more than 10 MU/g.

III. CAUSATIVE AGENT: TTX

A. DISTRIBUTION OF TTX-BEARING ORGANISMS

1. Puffer

It is well known that puffer generally contains TTX. TTX intoxication in humans most often results from ingestion of the liver of certain toxic species. Takahashi and Inoko (1889a,b) first attempted to study the chemical and physical properties of TTX partially purified from puffer. Later, TTX was successfully purified from the ovaries of puffer *Spheroides rubripes*. The structure of TTX was, however, approved in 1964 at the Fourth International Symposium on the ''Chemistry of Natural Products'' held in Japan, with the molecular formula of $C_{11}H_{17}N_3O_8$. Subsequently, the adopted term TTX attracted the attention of toxicologists, chemists, and pharmacologists, particularly for human intoxication.

Records of puffer poisoning have been described in the ancient literature from various parts of the world, particularly in Japan and China (Halstead, 1965; Kainuma and Baba, 1984). In Japan, the oldest record of puffer poisoning is found in Nara and Heian eras (800 AD).

Puffer frequently appeared in ''senryu,'' ''haiku,'' and poetry in the Edo era (1603–1868). These literatures indicate that puffer was a popular food item in those days. In China, puffers were eaten and caused human intoxication since the ancient time, nearly 2000 years ago.

Tani (1945) extensively studied the toxicity of Japanese puffer. Of 21 species assayed, 14 were toxic, such as *Takifugu porphyreus* (purple puffer), *Takifugu rubripes* (tiger puffer), *Takifugu pardalis* (panther puffer), *Takifugu obscurus* (''mefugu''), *Takifugu flavidus* (towny puffer), *Takifugu snyderi* (vermiculated puffer), *T. poecilonotus* (finepatterned puffer), *Takifugu chrysops* (purple puffer),

T. niphobles (grass puffer), *Takifugu xanthopterus* (striped puffer), *Takifugu stictonotus* (spotty back puffer), *Takifugu pseudommus* (“radamashi”), *Lagocephalus inermis* (smooth-backed blowfish), and *Canthigaster ribulata* (scribbled toby). In addition to these species, *Takifugu vermicularis* (pear puffer), *Tetraodon alboreticulatus* (“shiroamifugu”), *Takifugu chinensis* (eye spot puffer), *Lagocephalus sceleratus* (slack-skinned puffer), *Chelonodon patoca* (milkspotted blaasop), and *Takifugu exascurus* (“mushifugu”) were identified as TTX-bearing puffer.

The toxicity of 23 species of Taiwanese puffers was later studied by Hwang *et al.* (1992a). Only two puffers, *Lagocephalus gloveri* (brown-backed toadfish) and *Lagocephalus wheeleri* (brown-backed toadfish), are nontoxic in all tissues and may be used as materials for producing dried dressed fish fillets. But *Lagocephalus lunaris* (green toadfish), *T. oblongus* (oblong toadfish), and *T. niphobles* are the most toxic in all tissues and occasionally cause food poisoning incidents in Taiwan (Table VI). Later, Lin *et al.* (2002b) reported that the imported puffer *Tetraodon ocellatus* contained moderate amounts (100–999 MU/g) of toxin in skin and viscera and another one *Tetraodon nigroviridis* contained weak amounts (10–99 MU/g) of toxin in skin. The toxin from both species was composed of TTX and anhydroTTX.

In Japan, the toxicity of puffer is related to their spawning season, with the highest toxicity levels occurring between March and June, though the pattern varies to some degree among species. The toxin is concentrated in the ovaries, liver, and often the skin, making the female puffer more toxic than the males, especially during spawning season. The parts of the puffer considered less

TABLE VI
THE TOXICITY OF TAIWANESE PUFFERS

Fish species	Ovary	Testicle	Liver	Gall	Skin	Intestine	Muscle
Lagocephalus gloveri	—	—	—	—	—	—	—
Lagocephalus wheeleri	—	—	—	—	—	—	—
Lagocephalus inermis	⋆⋆	⋆	⋆⋆	⋆⋆	⋆	⋆⋆	—
Lagocephalus lunaris	⋆⋆⋆	⋆	⋆⋆⋆	⋆⋆	⋆⋆	⋆⋆	⋆⋆
Takifugu xanthopteus	⋆⋆⋆	—	⋆⋆⋆	⋆⋆	⋆	⋆⋆	—
Takifugu oblongus	⋆⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆	⋆⋆	⋆⋆
Takifugu flavidus	⋆⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆	⋆	⋆
Takifugu alboplumbeus	⋆⋆⋆	—	⋆⋆	⋆⋆⋆	⋆	⋆⋆	⋆
Takifugu niphobles	⋆⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆	⋆⋆	⋆

⋆⋆⋆, much higher toxicity, death after ingesting less than 10 g; ⋆⋆, high toxicity; ⋆, low toxicity; —, nontoxicity.
Source: Hwang *et al.* (1992a).

toxic are the musculature, fins, and testes. Although muscle tissue may be toxic in at least three puffer species, *T. pardalis, L. lunaris*, and *C. patoca*, no fatal cases of tetrodotoxication have yet been reported due to eating the meat of puffer as "sashimi," or sliced raw fish, in Japan. On the other hand, the dishes known as "chiri," fillets partially cooked in a stew of skins, livers, intestines, or testis, and "kimo," partially cooked livers, have been associated with much fatal intoxication.

Japanese toxic puffer, especially the species from northern parts generally contains an elevated level of TTX in the liver and gonad, while *C. patoca* inhabiting Okinawa and south of Amami-Oshima Islands, the subtropical part of the country, contains the highest toxicity in the skin (Khora *et al.*, 1991) and comparatively high toxicity in ovary, muscle, and testis (Mahmud *et al.*, 2001). Hashimoto (1979) described that the established toxicological knowledge on puffer of the Ryukyus and Amami-Oshima Islands indicating the possible environmental effect on their toxicity.

Recently, each toxin of Thai brackish water puffer *T. nigroviridis* and *Tetraodon steindachneri* has been characterized as same TTX. Toxin distribution pattern was similar to that of *C. patoca*. TTX as the toxic principle was also detected in the marine puffers from Taiwan and Bangladesh (Hwang *et al.*, 1988; Mahmud *et al.*, 1999a,b, 2001).

Seasonal individual and local variations of toxicity and toxin composition in puffer are occasionally observed. For example, panther puffer (*T. pardalis*) collected from the northern Pacific Coast of Japan exhibited much higher toxicity in the liver (mean 935 MU/g) compared to that form the southwestern part (337 MU/g). Puffers *Arothron mappa, Arothron manilensis, C. patoca, Arothron nigropunctatus, Arothron hispidus, Arothron Stellatus*, and *Arothron reticularis* collected from the Philippine water showed a remarkable individual difference in toxicity (Sato *et al.*, 2000). With a few exceptions, considerable amounts of STXs were detected in these species as well as in the ovary of Japanese marine puffer *Arothron firmamentum* (Nakashima *et al.*, 2004), although puffers inhabiting Japan contained mainly TTX. The toxic principle of the Thai freshwater *Tetraodon fangi* was reported as TTX (Laobhripatr *et al.*, 1990), but later the major toxin was identified as STX (Sato *et al.*, 1997). It is suggested that either TTX or PSP is overwhelmingly dominant in puffer.

Besides TTX, some researchers reported the presence of TTX derivatives in puffer. Nakamura and Yasumoto (1985) first isolated and identified tetrodonic acid (TDA), 4-*epi*TTX and anhydroTTX from *T. pardalis* and *T. poecilonotus*. Subsequently, 11-*nor*TTX-6(*R*)-ol, 6-*epi*TTX, and 11-*deoxy*TTX from *T. niphobles* and 11-*nor*TTX-6(*S*)-ol from *A. nigropunctatus* were isolated and characterized (Endo *et al.*, 1988; Yotsu *et al.*, 1992a).

2. Newts

Unexpectedly, TTX was detected in the eggs of a newt (*Taricha torosa*) by Mosher *et al.* (1965), which was the first report of the occurrence of this toxin in the animals other than puffer. Later, TTX was detected also in several species of newts from various countries (Wakely *et al.*, 1966). In Japan, there are three species of newts—*Cynops pyrrhogaster, Cynops ensicauda*, and *Tylototiton andersoni.* Among them, *C. ensicauda* from Okinawa and *C. pyrrhogaster* from main Japan were reported with special emphasis on their toxin profiles. TTX and its analogues 4-*epi*TTX, 6-*epi*TTX, 4,9-anhydro-6-*epi*TTX, 11-*deoxy*TTX, 11-*deoxy*-4-*epi*TTX, and 4,9-anhydroTTX were detected from *C. ensicauda* (Yasumoto *et al.*, 1988). *C. pyrrhogaster* toxin was found to consist of TTX, 6-*epi*TTX, and 11-*deoxy*TTX (Yotsu *et al.*, 1990a,b). However, *T. andersoni* was shown as a nontoxic species. Yotsu *et al.* (1990a,b) also reported the occurrence of 6-*epi*TTX and 11-*deoxy*TTX in a few species of newts *Taricha granulosa, Taricha oregon, Triturus vulgaris, Notophthalmus viridescens*, and *Ambystoma tigrinum* from the United States, in *Paramesotriton hongkongensis* from China, and *Triturus alpestris* from Italy. Toxin concentrations, measured by a fluorometric HPLC analyzer, were higher in *T. granulosa* (882 MU/g) and *N. viridescens* (673 MU/g) among them.

A total of 382 specimens of the Japanese *C. pyrrhogaster* were collected from western Japan and assayed for toxicity and toxin profiles (Tsuruda *et al.*, 2001). Most of them showed toxicity scores ranging from 5 to 370 MU/g. Among the parts, the skin and muscle showed higher toxicity score (56 MU/g) than the liver, stomach, intestine, and gonad whose scores ranged from less than 2 to 33 MU/g. Little seasonal, but large gender and regional variations of toxicity were recognized (Table VII). *C. pyrrhogaster* toxin consists of TTX and 6-*epi*TTX as the main components, and of 4-*epi*TTX, 4,9-anhydro-6-*epi*TTX, and 4,9-anhydroTTX as the minor ones. The marked interspecies differences in toxin profile may reflect their metabolic pathway of accumulated TTX.

3. Gobies

Goby toxin was isolated as crystalline state from the goby *Yongeichthys criniger* inhabiting the Amami-Oshima and the Ryukyu Islands and was identified from IR, ^{1}H NMR, and elemental analysis as TTX (Noguchi and Hashimoto, 1973). There was a rumor in these areas that farmers used to treat the dry goby as a rodent killer in the fields. In the subsequent study, goby fish was found to be toxic (Noguchi *et al.*, 1971). TTX concentration was generally high in the skin (with fins), followed by the viscera and muscle. In a few toxic specimens, the highest toxicity was in the mature testis. A rather wide local variation of toxicity was sharply observed (Table VIII).

TABLE VII
LOCAL AND SEXUAL VARIATION OF TOXICITY IN A JAPANESE NEWT *C. PYRRHOGASTER*

Place of collection	Date of collection	Sex[a]	Toxicity (MU/g)	
			Range	Mean ± S.D.[b]
Kishiku	April 1997	♂	6–11	8 ± 2
		♀	<5–35	15 ± 13
Nagasaki	May 1998	♂	12–160	72 ± 47
		♀	12–250	96 ± 63
Togitsu	September 1997	♂	<5–68	24 ± 28
		♀	<5–240	120 ± 103
Ohseto	June 1997	♂	64–140	83 ± 33
		♀	5–37	21 ± 12
Isahaya	July 1997	♂	67–210	127 ± 52
		♀	27–370	163 ± 138
Ohmura	May 1997	♂	<5–42	12 ± 17
		♀	6–33	18 ± 12
Nakabaru	June 1997	♂	9–17	12 ± 3
		♀	24–59	50 ± 15
Kiyama	June 1997	♂	9–10	10 ± 1
		♀	<5	0 ± 0
Gounoura	May 1997	♂	<5–29	9 ± 3
		♀	<5–13	4 ± 6
Houjou	May 1999	♂	15–150	77 ± 49
		♀	32–120	68 ± 37

[a]Five specimens were assayed for each sex.
[b]Mean ± S.D. was calculated on the assumption that toxicity of less than 5 MU/g was zero.
Source: Tsuruda *et al.* (2001).

Several fatal poisoning incidents and frequent deaths of duck, both due to ingesting the goby occurred in Taiwan (Lin *et al.*, 1996; Yang, 1967). An acute food poisoning due to ingestion of the gobies *Y. nebulosus* and *Sillago japonica* occurred in southern Taiwan, affecting two male persons (Lin *et al.*, 1999). TTX was identified as the causative agent of this food poisoning. The highest toxicity scores were 7650 and 1460 MU per specimen of *Y. nebulosus* and *S. japonica*, respectively. The toxicity of 12 species of Taiwanese gobies was examined and the specimens of three species of gobies, *Y. nebulosus, Prachaeturichtys palynena*, and *Radigobius caninus* were found to be toxic. The highest toxicity of *Y. nebulosus* was 4998 MU per specimen (Lin *et al.*, 2000).

4. Frogs

The discovery of TTX in newts and goby fish tempted scientists to search for new TTX-bearing animals. In 1975, TTX unexpectedly detected from the skin of Costa Rican frogs (average weight 1.3–1.8 g) *Atelopus varius varius,*

TABLE VIII
LOCAL VARIATION IN TOXICITY OF THE GOBY COLLECTED FROM JAPAN AND TAIWAN

Place of collection		Number of specimens tested	Toxicity (MU/g)	
Island/Country	Site		Range (MU/g)[a]	Mean ± S.D.
Amami	Tekebu	35	<20–160	64.9 ± 5.5
	Ikuma	19	<20–150	38.9 ± 9.7
	Shinokawa	20	<20–40	8.0 ± 3.0
Iriomote	Nakamagawa	11	<20–20	5.5 ± 2.8
Ishigaki	Naguragawa	5	<20	–
	Hukidougawa	15	–	66.7[b]
Taiwan		24	<20–120	30.0 ± 6.2

[a]The value <20 MU/g is calculated as 0 MU/g.
[b]Fifteen specimens were combined and assayed.
Source: Noguchi *et al.* (1971).

Atelopus varius ambulatorius, and *Atelopus chiriquiensis* (Kim *et al.*, 1975). Toxicity in the skin ranged from 100 to 120 MU per frog. *A. chiriquiensis* toxin consisted of a mixture of TTX (~30%) and chiriquitoxin whose structure replaces –C(12)-CH_2OH in TTX with –$CH(OH)CH(NH_3^+)COO^-$. Pavelka *et al.* (1977) found TTX and TTX-like compound in the eggs remained in a bound form. In 1992, TTX and its derivatives 4-*epi*TTX and 4,9-anhydroTTX were detected in the toad *Atelopus oxyrhynchus* from Venezuela (Yotsu *et al.*, 1992b). This species is able to retain a high level of TTX and its analogues in long captive position under artificial environment. Besides its occurrence in the frogs of the family Bufonidae, TTX and TTX-like substance were detected in the Panamanian frog *Colostethus inguinalis* (Family: Dendrobatidae) (Daly *et al.*, 1994) and Brazilian frog *Brachycephalus ephippium* (Family: Brachycephalidae) (Sebben *et al.*, 1986), respectively. The skin extract from *C. inguinalis* showed 0.05–6 MU TTX-equivalents per individual frogs in the mouse assay, showing a remarkable variation in toxin concentration. As described above, TTX-bearing frogs/toads had been confined to certain species of genus *Atelopus* (Family: Bufonidae), *Brachycephalus* (Family: Brachycephalidae) and *Colosthetus* (Family: Dendrobatidae) from South and Central America (Daly *et al.*, 1994; Kim *et al.*, 1975; Mebs and Schmidt, 1989; Mebs *et al.*, 1995; Pavelka *et al.*, 1977). TTX was detected also in the skin of rhacophoridid frog *Polypedates* sp. from a subtropical country (Tanu *et al.*, 2001). In the mouse assay, the skin toxicity ranged from 31 to 923 MU/g. Bigger specimens generally showed higher toxicity in comparison with that of smaller one and a wide local variation of toxin concentration was observed. In the skin extract, TTX was found by HPLC, electrospray ionization-time of flight/mass spectrometry

(ESI-TOF/MS), and ^{1}H NMR. In ESI-TOF/MS analysis, protonated molecular ion peak $(M + H)^{+}$ of the toxin appeared at $m/z = 320.1103$, suggesting the molecular weight to be 319.1025, the same as that of authentic TTX ($C_{11}H_{17}N_3O_8 = 319.10116$).

The skins of previously described *Atelopus* frogs from northern Panama and Costa Rica contain TTX and another chemically distinct but related toxin (chiriquitoxin). Kim *et al.* (2003) reported that *Atelopus zeteki*, a bright yellow-colored frog with variable black markings and only in a single local region (Valle de Anton) in Panama, was found to contain a new toxin (zetekitoxin). The molecular weight was determined by high-resolution electron spray ionization mass spectra (HRESIMS) (50% MeOH) $[M + H]^{+}$, *m/z*, 553.1369. The observed fragmentation $[-SO_3 + H]^{+}$, *m/z*, 473.1725 suggested the presence of $O–SO_3H$ or $N–SO_3H$ group. NMR experiments TOCSY, HSQC, $^{13}C–^{1}H$, and $^{15}N–^{1}H$ HMBC revealed the STX backbone structure. The N7 substitution is $CH_2O–CO–NHOH$. The amino group of this carbamate was determined by the fragmentation pattern to be hydroxylated. Other substitution moieties are suggested by rotating frame overhause effect spectroscopy (ROESY) and nuclear overhauser enhancement spectroscopy (NOESY). The C11 substitution is OSO_3H and other moieties of C6 and C11 are suggested to form fused bicyclic amide ring. Zetekitoxin is a new STX derivative which has a higher molecular weight than that of STX (Kim *et al.*, 2003).

5. *Horseshoe crab*

In the spawning season, female specimens of the horseshoe crab possess a large amount of eggs which Thai people are fond of eating, and which result in sporadic outbreak of poisoning (Banner and Stephens, 1966; Kanchanapongkul and Krittaya, 1995; Trishnananda *et al.*, 1966). There are two species of horseshoe crab *C. rotundicauda* and *Tachypleus gigas* inhabiting Thailand. The causative species was identified as *C. rotundicauda* in all the poisoning incidents. Sometimes, *T. gigas* is mistaken for *C. rotundicauda*, resulting in poisoning incident (Banner and Stephens, 1966).

Both are very similar to each other. The morphological differences between the toxic species (*C. rotundicauda*) and nontoxic one (*T. gigas*) are as follows: total length is approximately 300 mm in *C. rotundicauda* and 450 mm in *T. gigas*. The surface of the tail is smooth in the former while equipped with many prickles in the latter. Cross section of the tail is round in the former while triangle in the latter. The symptoms of horseshoe crab poisoning are similar to those of paralytic shellfish poisoning or TTX intoxication. The causative agent of poisoning was characterized as PSP (Fusentani *et al.*, 1982, 1983). In 1987,

however, the toxin of the same species from Thailand was shown to consist of TTX as the major toxin, with the highest score of 16 MU/g egg (Kungsuwan *et al.*, 1987).

Tanu and Noguchi (1999) reported the occurrence of TTX in the same species from Bangladesh. Most of 39 specimens collected were toxic. Among the tissues, the egg showed the highest toxicity, ranging from 2.0 to 7.4 MU/g (Table IX). Toxicity scores of the testis and viscera were less than 5 MU/g. The eggs showed a higher toxicity than the testis. However, the toxicity level in all tissues of Bangladeshi specimens was lower than the quarantine limit for human (10 MU as TTX per gram edible part). As a matter of fact, there has been no official record of horseshoe crab, and also no outbreak of poisoning.

It is assumed that TTX is involved in a defense mechanism to protect the horseshoe crab eggs from predators (Jeon *et al.*, 1984; Sheumack *et al.*, 1984). Horseshoe crab mainly feeds on mollusks, arthropods, and detritus (Chatterji *et al.*, 1992). Some bacteria inhabiting the marine sediments might be the primary origin of TTX for this crab.

6. *Xanthid crabs*

The occurrence of TTX in xanthid crabs *Atergatis floridus, Atergatopsis germaini, Zosimus aeneus, Lophozozymus pictor, Demania reynaudi*, and *Eriphia sebana* expands the diversity of TTX-bearing animals. The toxin composition of xanthid crabs *A. floridus* and *Z. aeneus* widely differed depending on the habitat. These two species had been known to possess only PSP (Daigo *et al.*, 1985; Koyama *et al.*, 1981; Yasumoto *et al.*, 1981). Noguchi *et al.* (1969) isolated crabtoxin from *Z. aeneus* in pure state and characterized as STX from its IR, specific rotation, specific toxicity, and TLC. Noguchi *et al.* (1983) first detected TTX as the major toxin (90%) in *A. floridus* collected from Miura Peninsula near Tokyo. In 1986, TTX was again reported as the major toxin in *A. floridus* collected from Kojima of Ishigaki Island in Okinawa, Oita, and Kumamoto Prefecture, with relatively low toxicity levels (38–80 MU/g) (Noguchi *et al.*, 1986a,b). In early 1990s, two novel TTX analogues 11-*oxo*TTX and 11-*nor*TTX-6(*R*)-ol were detected in *A. floridus* collected from the former Okinawa (Arakawa *et al.*, 1994).

In *Z. aeneus* specimens from the Philippines, TTX was identified as a minor toxin, with PSP as the main (Arakawa, 1988). Out of 32 specimens, 29 were toxic, with the toxicity scores of 14–850 MU/g. In this connection, *Z. aeneus* was examined for resistibility against PSP and TTX. The MLD of TTX in this species was estimated to be 1000–2000 MU/20 g body weight, in a strong contrast to the MLD (<1 MU/20 g) in nontoxic species (Koyama *et al.*, 1983).

TABLE IX
INDIVIDUAL TOXICITY OF HORSESHOE CRAB *C. ROTUNDICAUDA* FROM BANGLADESH

Date of collection	Specimens number	Sex	Body weight (g)	Toxicity (MU/g)		
				Egg	Testis	Viscera
November 1998	1	♂	121	—	2.6	2
	2	♂	172	—	3.9	2.5
	3	♀	275	7.0	—	3
	4	♀	260	5.3	—	2.2
December 1998	1	♂	190	—	4.8	2.8
	2	♀	255	6.7	—	2.8
	3	♀	232	6.6	—	3.9
	4	♀	249	7.4	—	3.2
	5	♀	243	6.2	—	2.9
	6	♀	237	5.4	—	3.3
	7	♀	223	5.8	—	2.7
	8	♀	241	5.2	—	3.5
	9	♀	254	6.2	—	3.8
October 1999	1	♂	180	—	3.8	<2
	2	♂	122	—	4.2	<2
	3	♂	158	—	<2	<2
	4	♀	237	2.6	—	<2
	5	♀	246	2.7	—	<2
	6	♀	275	3.2	—	<2
	7	♀	210	3.7	—	<2
	8	♀	190	3.8	—	<2
	9	♀	221	2.8	—	<2
	10	♀	186	3.8	—	<2
	11	♀	240	<2	—	<2
	12	♀	250	4.0	—	<2
	13	♀	265	3.3	—	<2
November 1999	1	♂	187	—	<2	<2
	2	♂	190	—	<2	<2
	3	♂	210	—	3.6	<2
	4	♂	168	—	4.2	<2
	5	♂	180	—	2.6	<2
	6	♀	230	3.8	—	<2
	7	♀	220	4.4	—	<2

Source: Tanu and Noguchi (1999).

The presence of TTX in *Z. aeneus* irrespective of the habitat, in addition to its resistibility against TTX, suggests that this toxin is essential for this species to survive.

In Taiwan, xanthid crabs *A. floridus, A. germaini, D. reynaudi, Z. aeneus, Xanthias lividus*, and *L. pictor* were reported as TTX as well as PSP-bearing

TABLE X
TOXICITY AND TOXIN COMPOSITION OF SIX TOXIC CRAB SPECIES COLLECTED FROM TAIWAN

Species	Toxin composition	Approximate toxicity (MU/g)	Collection place
Zosimus aeneus	TTX (82) GTX1–4 (18)	7	Lanyu, Wanlitung Hsiaoliuchiu
Lophozozymus pictor	TTX (89) GTX1, 3 (11)	5	Keelung
Atergatopsis germaini	GTX (50) hySTX (40) STX (7) TTX (3)	40	Keelung
Atergatis floridus	TTX (85) GTX1–4 (15)	8	Hsiaoliuchiu
Demania reynaudi	TTX (88) GTX2–4, neoSTX (12)	3	Keelung
Xanthias lividus	TTX (83) GTX1–4 (17)	6	Lanyu, Hsiaoliuchiu

Data in parentheses represent the percentage.
Source: Hwang and Tsai (1999) and Tsai *et al.* (2002).

crustaceans (Table X) (Hwang, 2003a,b; Hwang and Tsai, 1999; Tsai *et al.*, 1995, 1996, 1997a,b, 2002). In *A. floridus*, 85% of the total toxicity was accounted for TTX and 15% by GTX1–4, whereas in *A. germaini*, 3% by TTX, 50% by GTX3, 7% by neoSTX and STX, and 40% by an unknown toxin. In *D. reynaudi*, 88% by TTX and 12% by GTX2, GTX4, and neoSTX, *L. pictor* occasionally has been causing human intoxication in Taiwan as well as Singapore, whose toxin was identified as TTX in the former country and palytoxin in the latter. In *Z. aeneus* and *X. lividus*, 82–83% of the total toxicity was accounted for TTX and 17–18% by GTX1–4. The toxic composition and toxicity in toxic crabs collected from different areas are shown in Table XI (Hwang and Tsai, 1999).

7. *Blue-ringed octopus*

Blue-ringed octopus *Hapalochlaena* (*Octopus*) *maculosus* (Family: Octopodidae) has so far been identified as the only lethal and TTX-bearing octopus. It is found in shallow coral and rock pools in the waters around Japan, Taiwan, Philippines, and Australia. This octopus probably produces a poison in the salivary glands, which seems to be used for hunting and defense purposes. Many researches attempted to elucidate the causative agent of human fatalities attributed to the bite from this species. Although, it was initially claimed to be the new toxin,

TABLE XI
TOXIC COMPOSITION AND TOXICITY IN TOXIC CRABS

Species	Toxin	Approximate toxicity (MU/g)	Place
Xiphosuridae			
Carcinoscorpius rotundicauda	PSP	40	Thailand
Coenobitidae			
Birgus latro	TTX	10	Thailand
	Unknown	1	South Pacific Ocean Ryukyu
Xanthidae			
Zosimus aeneus	STX, neoSTX, dcSTX, GTXs	2000	Ryukyu
	STX, neoSTX, GTXs	20	Australia
	STX, neoSTX, GTXs, TTX	220	Philippines
	PSP, TTX	20	Philippines
	STX, neoSTX, GTXs	9	Fuji
	PSP	50	Palau Island
	TTX, GTX1–4 (minor)	7	Taiwan
Atergatis floridus	STX, neoSTX	1400	Ryukyu
	TTX and related compounds	20	Ishigashi Island
	TTX, STX (minor)	50	Miura Peninsula, Japan
	GTXs	180	Chiba, Japan
	STX, neoSTX, GTX2 (minor)	63	Fuji
	TTX	10	Australia
	TTX, GTX1–4 (minor)	8	Taiwan

Platypodia granulosa	70% STX, 30% unknown toxin	400	Ryukyu
Lophozozymus pictor	Palytoxin	1400	Philippines
	GTX2	5	Australia
	Isomer of palytoxin	6000	Singapore
Atergatopsis germaini	TTX, GTX1–3 (minor)	5	Taiwan
	STX, GTX, TTX (minor)	40	Taiwan
Demania alcala	Palytoxin	3000	Philippines
Demania reynaudi	Palytoxin-like	8	Philippines
	TTX (minor), GTX2–4, neoSTX,	3	Taiwan
Demania toxica	Unknown	100	Philippines
Eriphia sebana	STX, neoSTX, GTX1, 2	9	Australia
Eriphia scabricula	STX, neoSTX, GTXs	16	Ryukyu
Leptodius sanguineus	PSP	2	Australia
Neoxanthias impressus	PSP	4	Ryukyu
Pilumnus vespertilio	STX, neoSTX, GTXs	3	Ryukyu
Portunidae			
Thalamita sp.	PSP or GTX1	4	Australia, Ryukyu
Grapsidae			
Grapsus albolineatus	PSP	1	Australia, Ryukyu

Source: Hwang and Tsai (1999).

maculotoxin (Sutherland and Lane, 1969), finally it was identified as TTX (Sheumack and Howden, 1978). Its bite can paralyze the muscle much quicker than eating the puffer. The rapid response might be due to the prompt action of the intruded toxin to the neuron, in comparison to ingested toxin that ultimately affects the brain through a long gastrointestinal pathway. An adult human may be killed within 20 minutes after the bite, if not treated immediately and properly. The toxin was found to exist not only in the salivary gland but also in the ovary of this octopus (Hwang *et al*., 1989; Sheumack *et al*., 1984).

All octopuses generally have ink sac where a lot of ink is pooled. When they meet enemy or are attacked by them, they secrete a lot of ink from their sac and can conceal themselves to escape. Since they have a strong defense substance of TTX and need not use ink, the sac has lost above function. Significance why this blue-ringed octopus has TTX seems to use TTX for capturing preys or defensing itself from enemy.

8. Gastropods

In December 1979, a paralytic poisoning by ingesting the digestive gland of trumpet shell *C. sauliae* (Family: Cymatiidae) occurred in Shizuoka Prefecture, Japan. The responsible toxin of poisoning was identified as TTX, and this was the first report of the occurrence of TTX in a gastropod (Narita *et al*., 1981). Since then, TTX was also detected in other gastropods such as *Tutufa lissostoma, Natica lineata, N. vitellus, Rapana rapiformis*, and *R. venosa venosa* from Japan and Taiwan (Cheng *et al*., 1996; Hwang *et al*., 1990a, 1991a,b,c, 1992a,b,c,d, 1994b; Jeon *et al*., 1984; Narita *et al*., 1984; Noguchi *et al*., 1981; Yasumoto *et al*., 1981). In 1980, specimens of Japanese ivory shell, *Babylonia japonica* (Family: Buccinidae) were collected from Fukui Prefecture, Japan and assayed (Noguchi *et al*., 1981). The highest toxicity (as TTX) recorded was 53 MU/g digestive gland. In 1981 and 1982, 22 of *T. lissostoma* (Family: Bursidae) specimens were collected in Shizouka Prefecture, 17 of them were toxic (Noguchi *et al*., 1984). Toxicity was detected in the digestive gland exclusively, with the highest score of 700 MU/g. The occurrence of TTX and anhydroTTX in a gastropod mollusk *N. lineata* (lined moon shell) was reported by Hwang *et al*. (1990a). The highest toxicity (720 MU/g) was found in the muscle, while the other parts including digestive gland were much less toxic (12–28 MU/g). Specimens showed a wide individual variation in toxicity. The other small toxic gastropods in Taiwan, including *N. vitellus, Polinices tumidus*, and *Polinices didyma*, contain higher amounts of TTX in the muscle, following with digestive gland and other tissues (Table XII). The gastropods *Z. scalaris, N. clathrata*, and *Zeuxis sufflatus* contain TTX in all tissues. *N. clathrata* also contain minor amounts of PSP in the spring. On the other hand, the small

TABLE XII
THE TOXICITY AND TOXIN COMPOSITION OF SEVERAL GASTROPODS COLLECTED

Species	Toxin composition	Approximate toxicity (MU per specimen)	Main toxin distribution	Collection place
Natica vitellus	TTX	20	Muscle	Kaohsiung
	TTX	14	Muscle	Pingtung
Natica alapailionis	TTX	6	Viscera	Pingtung
Natica lineata	TTX	93	Muscle	Kaohsiung
	TTX, PSP	47	Muscle	Pingtung
	TTX	21	Muscle	Chaiyi
Niotha clathrata	TTX	4	Viscera	Tainan
	TTX, PSP	139	Viscera	Pingtung
Zeuxis scalaris	TTX, PSP	4	Viscera	Pingtung
Zeuxis castus-like	TTX, PSP	42	Viscera	Pingtung
Oliva miniacea	TTX	146	Muscle	Pingtung
Oliva mustelina	TTX	35	Muscle	Pingtung
Oliva hirasei	TTX	58	Muscle	Pingtung
Babylonia formosae	TTX	1	Viscera	Pingtung
Rapana rapiformis	TTX	30	Viscera	Kaohsiung
Rapana venosa venosa	TTX	170	Viscera	Ilan

toxic gastropods *O. miniacea, O. mustelina*, and *O. hirasei* contain only TTX in the muscle, not in the digestive gland (Hwang, 2003a,b; Hwang *et al.*, 2003). The incidents of gastropod poisoning have occasionally occurred in Taiwan (Hwang *et al.*, 1995, 2002a,b, 2003, 2005; Shiu *et al.*, 2003).

Outbreaks of paralytic snail poisoning recently occurred in China. The epidemiological characteristics of this disease from an outbreak in Xhoushan City were recorded. Forty-two outbreaks of paralytic snail poisoning, involving 309 cases, occurred from 1977 to 2001 (Figure 3). Sixteen people (5.2%) died, 48 people (15.5%) required intubations, and 140 people (45.3%) required emergency hospital treatment as a result of these outbreaks. Outbreaks included multiple marine gastropod species, major species *Z. samiplicutus*, and occurred primarily during the summer (June–August) on 11 islands with high population densities. Peak numbers of outbreaks and amounts of gastropod toxicity occurred from 1978 to 1979 and from 1992 to 1994. Toxicity varied depending on specimen, region, and season. The toxin involved was identified as TTX (Shui *et al.*, 2003; Sui *et al.*, 2002).

9. *Starfish*

Noguchi *et al.* (1982) first identified TTX in a starfish *Astropecten polyacanthus*, suggesting that this species is involved in intoxication mechanism in the TTX-bearing trumpet shell *C. sauliae*. Subsequently, two starfish

Astropecten latespinosus and *Astropecten scoparius* were also added to the list of TTX-bearing echinoderms (Maruyama *et al.*, 1984, 1985; Miyazawa *et al.*, 1985) from the results of toxicity examination of starfish collected from all over Japan. In the mouse assay, *A. latespinosus* and *A. scoparius* showed the toxicity scores of 13 and 5.7 MU/g, respectively. These findings made sure of TTX intoxication mechanism in gastropods to come from their food chain. Afterward, TTX study gave a clue to be expanded in many aspects. In 1985, TTX was also reported from starfish *A. polyacanthus* and *A. scoparius* from Seto Inland Sea, Japan, with the highest toxicity scores of 520 and 46 MU/g, respectively (Miyazawa *et al.*, 1985).

A. scoparius was found to contain TTX in Taiwan as well, with the highest toxicity of 354 MU/g in the viscera (Lin *et al.*, 1998b). Toxin was characterized by HPLC and GC–MS analysis. GTX2–3 and STX (12%) were detected as the minor toxins. Main TTX intoxication mechanism of these carnivorous starfish is estimated to come from their food webs (Lin and Hwang, 2001). The toxicity of starfish was suddenly increased in September and reached to the maximum level in November. The highest level of toxicity was 16,821 MU per specimen. It was found that a significant amount of the toxin was there in the gonad of starfish. The starfish matured between October and November. The toxicity of gonad was increased as the maturity increases and reached to the top in November. The preyed animals in the digestive gland of starfish *A. scoparius* were mainly mollusks Bivalvia and Gastropoda. The amount of Gastropoda was marked by increase in number in October and November. The starfish *A. scoparius* might mainly accumulate high amount of TTX from smaller gastropod *Umborium suturale* and *Natica pseustes*. Furthermore, the starfish *Astropecten vappa* is reported to contain only TTX in Taiwan (Tsai *et al.*, 2004).

10. Flatworms

Miyazawa *et al.* (1986, 1987) first reported the occurrence of TTX in marine flatworms. Ten specimens of a flatworm *Planocera multitentaculata* were collected from the identical zone of the Seto Inland Sea, Ehime Prefecture, Japan and studied. The toxin was characterized as TTX by TLC, electrophoresis, and GC–MS analyses. The average lethal potency was 300 MU/g. In the same year, another flatworm *Planocera reticulata* was demonstrated to have TTX (Jeon *et al.*, 1986). Anatomical distribution of TTX in *P. multitentaculata* from Hiroshima Prefecture, Japan was also examined (Asakawa *et al.*, 2003), and it was found that the oviduct showed the highest toxicity (2000–3420 MU/g), followed by digestive organs (1400 MU/g) and others. The eggs laid by the flatworm showed an extremely high lethal

TABLE XIII
TOXICITY OF EGGS LAID BY THE FLATWORM *P. MULTITENTACULATA*

Date of collection	Place of collection	Weight of egg masses (g)[a]	Toxicity (MU/g)	Mean toxicity of parental worm
May 8–18, 1985	Laboratory aquarium[b]	0.6	10,700	1160
May 6	Kata, Wakayama	0.5	980	170[c]
May 22	Iwashijima, Hiroshima	0.9	3660	1330[c]
May 22–June 1	Laboratory aquarium	3.4	3820	–
June 3	Iwashijima, Hiroshima	1.4	5600	700[c]
June 3–8	Laboratory aquarium	2.0	2530	–
June 16	Asanami, Ehime	1.3	7600	220[c]
June 22	Iwashijima, Hiroshima	0.6	8100	1210[c]
June 22–July 5	Laboratory aquarium	1.0	3810	1350[c]
July 5	Iwashijima, Hiroshima	0.06	1000	–
July 5–13	Laboratory aquarium	1.6	6600	–
			(Mean ± S.E.: 5760 ± 870)	

[a]Combined sample in most cases.
[b]The parental flatworms reared in the laboratory aquarium have been collected from Iwashijima, Hiroshima.
[c]Mean toxicity of flatworms collected from around the egg masses.
Source: Miyazawa *et al.* (1986).

potency (5760 ± 870 MU/g), with the highest score of 10,700 MU/g, which were 2–50 times higher than those of parental flatworm (Table XIII). The eggs could use such a high level of TTX for a defensive purpose against predators. Main TTX intoxication mechanism is also estimated to come from their food webs. In this case, the part of TTX in the flatworms is very much distinct to be a defense substance to prevent the eggs from predators.

In this connection, according to Chinese literature, Chinese toxic puffer feeds on big flatworms. This puffer may be intoxicated with TTX through this food chain. Lin *et al.* (1998a) reported that the specimens of ''torafugu'' *T. rubripes* cultured in inland planes were nontoxic, but those in the coastal areas were toxic in the period between January and March in Taiwan. The toxin was found only in the liver and ovary of puffer. The toxic flatworm *Stylochus orientalis* was found to be the toxin source of cultured puffer. Except *P. multitentaculata* and *S. orientalis, P. reticulata, Stylochus ijimai*, and *Koinostylochus* spp. have been reported containing a high amount of TTX, but other species as *Stylochoplana clara, Notoplana humilis*, and *Notocomplana koreana* exhibited a low or trace toxicity (Jeon, 1985).

11. Ribbon worms

The occurrence of TTX in flatworm led scientists to extensive studies on nemertines, resulting in detection of TTX for the first time in two species of ribbon worm *Lineus fuscoviridis* and *Tubulanus punctatus*, with the highest score of 503 and 540 MU/g, respectively (Miyazawa *et al.*, 1988). In addition to TTX, toxic TDA-like substance was detected from these ribbon worms. Kem (1976) isolated four polypeptides from the toxic ribbon worm *Cerebratulus lacteus* that showed a selective toxicity variety of animals, suggesting that the toxin is also used for a defensive purpose. Subsequently, the toxic mucus secretion of *C. lacteus* was shown to consist of cytotoxins and neurotoxins (Kem and Blumental, 1978).

Later, *Cephalothrix linearis* from Shizuoka Prefecture, Japan was calculated to be a TTX-bearing species (Ali *et al.*, 1990). The highest lethal potency was calculated to be 23,000 MU/g whole body, which is about 40 times greater than those scores of *L. fuscoviridis* and *T. punctatus* (Miyazawa *et al.*, 1988). In *C. linearis*, the toxin was densely distributed in the proboscis (22,000 MU/g), a special device to prey food animals, followed by the other parts (13,600 MU/g). Its mucus secretion also contained TTX at a high level. This species showed rather wide individual and seasonal variations in lethal potency. The ribbon worms have ability to secrete a considerable amount of toxin when stimulated, suggesting that they utilize TTX for both defensive and offensive purposes. The toxin secreted from *C. linearis* was mainly composed of a TDA-like substance, which showed same behavior as TDA in HPLC analysis. In this connection, Noguchi *et al.* (1991) purified a TDA-like substance from *C. linearis* and the flatworm *P. multitentaculata*, which exhibited a specific toxicity of 700 MU/mg. The TDA-like substance clearly differs from TDA which is completely nontoxic. However, mass number of TDA-like substance is 320, not same as that of authentic TTX (319 dalton), suggesting that the TDA-like substance is a precursor of TTX or tautomer with H^+. Between April 1998 and December 2001, during the surveillance of the toxicity of various marine fouling organisms in Hiroshima Bay, Hiroshima Prefecture, specimens of the ribbon worm *Cephalothrix* sp. showed very high toxicity of 25,590 MU/g whose main toxin was identified as TTX from various instrumental analyses. The component patterns agree with that of the *C. linearis* (Asakawa *et al.*, 2003).

12. Annelids

The occurrence of TTX, 4-*epi*TTX, and anhydroTTX in the annelid *Pseudopotamilla occelata* was detected by Yasumoto *et al.* (1989). During its investigation, the highest toxicity was recorded to be 24 MU/g. TTX as the

TABLE XIV
TOXICITY OF FOUR SPECIES OF ANNELID COLLECTED FROM THE SETO ISLAND SEA, JAPAN

Species	Body weight (g)	Highest toxicity (MU/g)	Ratio of toxic specimens
Lepidonotus helotypus	0.7–1.9	110	8/14
Halosydna brevisetosa	0.3–0.4	14	2/3
Hermenia acanthopeltis	0.3	20	1/1
Harmothoe imbricata	2.1	4	1/1

Source: Yasumoto *et al.* (1989).

major toxin was identified by HPLC and TLC methods. Later *Lepidonotus helotypus, Halosydna brevisetosa, Hermenia acanthopeltis*, and *Harmothoe imbricata* were also detected as TTX-bearing annelids (Table XIV). *L. helotypus* showed the highest score (110 MU/g), which was accounted for mainly by TTX and partly by the TDA-like substance.

13. *Arrowworms*

TTX was also detected in several species of marine arrowworm (zooplankton), such as *Eukrohnia hamata* (Family: Eukrohniidae), *Parasagitta elegans, Flaccisagitta scripassae*, and *Flaccisagitta enflata* (Family: Sagittae), and *Spadella angulata* (Family: Spadallidae) (Thuesen *et al.*, 1988). Arrowworms could utilize TTX to paralyze their food organisms. They are carnivorous in nature and represent a vital position in food web to intoxicate the higher animals.

14. *Red calcareous alga*

Paralytic toxicity was detected in a red calcareous alga *Jania* sp. from Okinawa, Japan and Gambier Islands, French Polynesia (Kotaki *et al.*, 1983; Yasumoto *et al.*, 1989). The highest toxin concentration was 44 ppb. The Okinawa *Jania* sp. contained anhydroTTX as the major toxin, along with TTX and 4-*epi*TTX as the minor, while the French one showed TTX as the major toxin. In the above red alga, TTX content is very low and therefore its origin may come from TTX-contaminated bacterium.

15. *Dinoflagellate*

Kodama *et al.* (1996) identified TTX in cultured cells of *Alexandrium tamarense*, a notorious PSP-producing dinoflagellate. TTX was identified by HPLC-fluoro metric analysis and fast atom bombardment mass spectrometry (FABMS).

TTX accounted for about 0.1% of the total toxicity of the dinoflagellate cells. However, the occurrence of TTX in *A. tamarense* should be rationalized more clearly.

16. Bacteria

During the last two decades a number of reports have been published, showing that many bacteria contained TTX. In 1986, the occurrence of TTX and anhydroTTX in *Vibrio* sp. from the intestines of a xanthid crab *A. floridus* was first demonstrated by Noguchi *et al.* (1986b). In its experiment, four strains of *Vibrio* sp. isolated from the contents of the intestines of *A. floridus* were grown in flask containing 500-ml water with 5 g each of phytone peptone and NaCl (pH 7.2), and incubate at 25°C for 10 days. The harvested cells from one strain showed a considerable amount of toxin (30 MU/flask). The presence of TTX and anhydroTTX in the cell extract was confirmed by HPLC. Ultraviolet (UV) spectrometry and GC–MS analysis indicated the presence of C_9-base in the alkaline decomposed compounds of the harvested bacterial cells. In the same year, Yasumoto *et al.* (1986) also isolated two bacteria *Shewanella alga* and *Alteromonas tetraodonis* from the red calcareous alga *Jania* sp. and cultured them. TTX was detected in their culture broth.

In 1987, *Vibrio alginolyticus* isolated from the starfish *A. polyacanthus* and puffer *T. snyderi* contained TTX (Narita *et al.*, 1987; Noguchi *et al.*, 1987). In the study of Noguchi *et al.* (1987), 26 of 33 strains of aerobic and facultative anaerobic bacteria that were isolated from the puffer were found to belong to genera of *Vibrio*. By instrumental analysis, TTX was detected in all strains of *V. alginolyticus*.

Simidu *et al.* (1987) cultured a number of strains of marine bacteria and screened for toxin by HPLC and GC–MS analyses. Results showed that 12 of the 24 strains, which included most strains of Vibrionaceae, produced TTX and/or related substances (Table XV).

Attempts were also made to isolate TTX-bearing bacteria from the blue-ringed octopus *O. maculosus* from the Philippines (Hwang *et al.*, 1989). The results showed that 16 of the 22 isolated strains produced TTX and/or related substances. Six of the 16 strains produced TTX and/or related substances. Six of the 16 strains were classified into the genera of *Vibrio, Pseudomonas*, and two each of *Alteromonas* and *Bacillus.*

The capability of TTX production by the bacteria *Shewanella putrefaciens* isolated from the intestine of a puffer *T. niphobles* was reported by Matsui *et al.* (1990). After being injected with the culture broth, the mice showed typical symptoms of TTX intoxication. A 25 ml of broth contained 15 MU of the toxin which was identified as TTX by GC–MS analysis.

TABLE XV
ANALYSIS OF TTX IN BACTERIAL CELLS

Bacterial strains used	HPLC		GC–MS
	Tetrodotosin	AnhydroTTX	
Vibrio alginolyticus ATCC 17749	–	+	+
Vibrio alginolyticus NCMB 1903	–	+	+
Vibrio anguillarum NCMB 829	–	+	+
Vibrio anguillarum NCMB1291	–	+	+
Vibrio costicola (*V. costicolus*) NCMB 701	–	+	+
Vibrio fischeri NCMB 1281	–	±	–
Vibrio fischeri (*Photobacterium fischeri*) NCMB 1381	–	±	±
Vibrio harveyi (*Aeromonas harveyi*) NCMB 2	–	±	+
Vibrio marinus Ps 207	–	±	±
Vibrio parahaemolyticus ATCC 17802	–	+	+
Vibrio parahaemolyticus NCMB 1902	–	+	+
Vibrio parahaemolyticus ATCC 17802	–	+	+
Photobacterium phosphoreum NCMB 844	±	+	+
Aeromonas hydrophila NCMB 89	–	+	–
Aeromonas hydrophila NCMB 89*d	–	±	–
Aeromonas salmonicida ATCC 14174	±	+	+
Aeromonas salmonicida ATCC 14174*d	±	±	+
Plesiomonas shigelloides ATCC 14029	±	+	+
Escherichia coli IAM 1268	–	±	–
Escherichia coli IAM 1268*d	–	±	±
Alteromonas communis IAM 12914	–	±	±
Alteromonas haloplanktis IAM 1218	–	–	–
Alteromonas nigrifaciens IAM 13010	±	±	±
Alteromonas undina IAM 12922	–	–	–
Alteromonas vaga IAM 12923	±	–	±

+, clearly detected; ±, difficult to detect; –, not detected; *d, cultivated in a freshwater medium.
Source: Simidu *et al.* (1987).

Hwang *et al.* (1994c) isolated bacteria representing four predominant genera *Vibrio, Aeromonas, Flavobacterium*, and *Pseudomonas* from the Taiwanese lined moon shell *N. lineata.* The genera *Vibrio* composed of more than 46% of the total population. Presence of TTX and/or related substances in *V. alginolyticus* and *Aeromonas* spp. was confirmed by HPLC, UV, and GC–MS analyses.

Later, some bacteria, such as *V. alginolyticus, Vibrio parahaemolyticus, Aeromonas* sp., *Pseudomonas* spp., and *Plsiomonas* sp. from a gastropod *N. clathrata*, were demonstrated to produce TTX and/or related substances (Cheng *et al.*, 1995). On the other hand, Kogure *et al.* (1988) examined some 50 strains of bacteria isolated from the marine sediments at 4000-m depths from Tokyo Bay, Japan. TTX concentration in the cells ranged from 0.13 to 0.43 MU/g wet sediment (Kogure *et al.*, 1988). Sediments collected at the 4000-m depth showed a higher amount of TTX. *V. alginolyticus* was reported to produce more amount of TTX when cultured at facultative anaerobic condition than aerobic condition (Lin, 1999).

The above findings indicate that marine bacteria belonging to the genus *Vibrio*, more specifically *V. alginolyticus*, are more frequently and abundantly available in toxic marine animals and more capable of producing TTX and/or related derivatives than others.

B. TTX ELABORATOR

In the past several decades we have made tremendous progress in the analysis, chemistry, pharmacology, and biology of TTX. The wide distribution of TTX in a variety of organisms, along with marked individual, regional, and seasonal variations in toxin concentration, led us to controversies of the endogenous or exogenous origin of TTX. Kim *et al.* (1975) pointed that the ability to synthesize TTX is a coincidental genetic development in certain animals. Yotsu *et al.* (1992b) demonstrated that the tree frog *A. oxyrhynchus* retained a high level of toxicity when raised in a controlled environment for more than 3 years. The skin extracts of the hatched-raised frog (*A. varius*) from the eggs demonstrated the presence of TTX (Daly *et al.*, 1997). Nagashima *et al.* (1999) examined subcellular distribution of TTX and its derivatives in the puffer liver and indicated the possible endogenous origin of TTX. However, the most important observation regarding individual, local, and seasonal variations of toxicity in TTX-bearing animals are not yet comprehensively rationalized by the endogenous theory. Because an animal produces toxin endogenously, there are usually no significant individual, local, and seasonal variations in toxicity. However, TTX-bearing animals are often found to show significant variations depending on the individual, location, and season.

In contrast, there have been a few attractive documents in favor of the exogenous origin of TTX. Meanwhile, several attempts were made to confirm the exogenous speculation and it was postulated that bacteria are responsible for the production of TTX (Noguchi *et al.*, 1987; Yasumoto *et al.*, 1986). In support of this postulation, two possible TTX intoxication routes have been described so far. First, some bacteria produce TTX in the sediment and then the toxin is transferred to higher animals through the food web. Second, the host animals accumulate TTX through the symbiotic way. These ideas have been generated and justified from the discovery of TTX-producing bacteria in various toxic host animals and in marine sediments as well, as described in the previous section.

There have been also a few documents containing different approaches in support of the exogenous (food chain) hypothesis. Toxicity of all livers and partly muscles, gonads, and other viscera of above 5000 puffer fish (*T. rubripes*) cultured for 1–3 years in surrounding nets in eight Japanese prefectures were found to be nontoxic. When nontoxic puffer fish produced were reared with each TTX-containing diet of 0.5 and 4 MU TTX per gram fish body per day, respectively, they became toxic in 100 days after dosage of 4 MU/g fish body per day. These results show that puffer fish are intoxicated from the food chain (Noguchi, 1988).

Debris of starfish was detected in the intestine of the trumpet shell *C. sauliae*, another TTX-bearing animal. Subsequently, Noguchi *et al.* (1982) demonstrated that the starfish, which the trumpet shell prefers as food, also contain TTX. The erratic distribution of TTX in *Atelopus* frog (Kim *et al.*, 1975) and goby (Noguchi *et al.*, 1971) suggested the possible accumulation through the food chain. In respect of different feeding habits of the diverse TTX-bearing animals living in various environments, however, TTX accumulation mechanism should be rationalized more clearly.

As illustrated in this chapter, number of TTX-bearing organisms has been increasing, especially since 1970. Despite the occurrence of TTX in a wide diversity, the mechanism of the induction of toxicity in TTX-bearing animals and the dynamic state of TTX and its analogues have not been completely described. However, considering that all TTX-bearing organisms live in sea or freshwater all or partly through their life, the mechanism of their TTX intoxication seems to be more or less involved in their ecological factor such as water. Information on the influence of environmental parameters, temperature, and water on toxin accumulation and toxin principle are also sorely lacking. Studies along these lines can be progressed.

In regard with transmission of TTX through the food web, TTX in puffers seems to come directly from their food, such as toxic gastropods, flatworm, and starfish. TTX commonly accumulates in the eggs of puffers,

presumably transmitted from the mother, since the toxin generally disappears from the larvae 1 week after fertilization. In the wild puffer, the toxin usually reappears from the larvae after about 2 weeks probably through ingestion of toxic organisms. Indeed, when larvae puffers initially possessing TTX during the first week after fertilization are cultured and fed a TTX-free diet, they do not become toxic. Thus, TTX intoxication mechanism in puffers is likely to occur through the food web, though intestinal bacteria in some species of these fish reportedly produce negligible amounts of the toxin. Puffers, however, seem inclined to seek TTX in their diets. Ingested toxin is initially accumulated in the liver, followed by the skin and other tissues. Fish, except those possessing TTX, such as puffers and the tropical goby *Y. criniger*, do not accumulate TTX even when toxin-containing diets are fed to them at sublethal doses (Ebesu *et al.*, 2000; Hwang, 2003a,b; Miyazawa and Noguchi, 2001; Noguchi *et al.*, 2004).

Other TTX-bearing animals such as gastropods and starfish probably accumulate their toxins through the food web as well. In the trumpet shell (*C. sauliae*), TTX is ingested through its prey. During elucidation on the TTX transmission mechanism for this animal, starfish such as *A. polyacanthus, A. latespinosus*, and *A. scoparius* on which the gastropod feeds were determined to be links in the food web leading to TTX intoxication (Ebesu *et al.*, 2000; Hwang, 2003a,b; Miyazawa and Noguchi, 2001). These toxic starfish are widely distributed in Japan. TTX-containing starfish probably derive the toxin through their diets. The ivory shell (*B. japonica*) is likely intoxicated with TTX by ingesting toxic puffers that fishermen discard in the sea.

TTX intoxication mechanisms through food web links have already been established by model experiments (Ebesu *et al.*, 2000; Hwang, 2003a,b; Miyazawa and Noguchi, 2001). From these studies, the intoxication method is hypothesized as illustrated in Figure 4. The initial source of TTX found in most animals, however, is believed to be marine bacteria. Toxic animals accumulate TTX through the food chain as the main route, minor from bacteria.

C. MECHANISMS OF TTX INFESTATION TO ANIMALS

Various toxic tetraodontid species are globally distributed (Ebesu *et al.*, 2000; Hwang, 2003a,b; Miyazawa and Noguchi, 2001). However, these fish are consumed only in certain parts of the world. Areas that have reported TTX poisoning cases due to consumption of toxic puffer include Japan, Taiwan, China, Hong Kong, Thailand, Korea, Singapore, Malaysia, Bangladesh, Australia, the United States, Kiribati, Papua New Guinea, and Fiji. Other organisms found to possess TTX are listed in Table II. Like puffers, these animals are found worldwide: Japan (gobies, blue-ringed octopuses, various

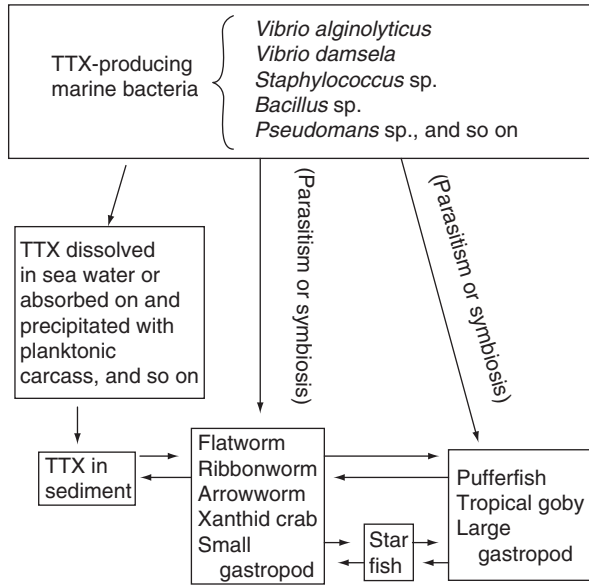


FIG. 4 Proposed mechanism of TTX accumulation in marine animals.

gastropods, starfish, xanthid crabs), Taiwan (gobies, various gastropods, xanthid crabs, starfish), Philippines (goby), Bangladesh (rhacophoridid frog, horseshoe crab), Costa Rica and Panama (*Atelopus* frogs), Australia (blue-ringed octopus), the United States (newts), and Thailand (horseshoe crab).

Puffers were long believed to be the exclusive source of TTX (Ebesu *et al.*, 2000; Hwang, 2003a,b; Hwang and Lu, 2003; Miyazawa and Noguchi, 2001). The toxicity of puffer is related to their spawning season, with the highest toxicity levels occurring between March and June for Japanese puffers, though the pattern varies to some degree among species. The toxin is concentrated in the ovary, liver, and often, the skin, making the female puffer more toxic than the males, especially during spawning season. The parts of the puffer considered less toxic are the musculature, fins, and testes. Although muscle tissue may be toxic in at least three puffers, *T. pardalis, L. lunaris*, and *C. patoca*, no fatal cases of tetrodotoxication have yet been reported due to eating the meat of puffer as sashimi, or sliced raw fish, in Japan. On the other hand, the dishes known as "chiri," fillets partially cooked in a stew of skins, livers, intestines, or testes, and "kimo," partially cooked livers, have been associated with much fatal intoxication.

The origin of TTX in TTX-bearing animals was often debated to be either endogenous or exogenous. In 1964, however, this toxin was unexpectedly

detected in the California newt (*T. torosa*) (Mosher *et al.*, 1965), thus, setting the controversy. In this organism, TTX was concentrated in the skin, ovary, muscle, and blood. Since then, TTX has been found in a variety of animals (Table II).

Other newt species, including *Taricha rivularis, T. granulosa, T. vulgaris, Triturus cristatus, T. alpestris, Triturus marmoratus, N. viridescens, C. pyrrhogaster*, and *C. ensicauda*, were also discovered to possess TTX in almost the same anatomical distribution as that of *T. torosa* (Wakely *et al.*, 1966; Yotsu *et al.*, 1990a). In 1971, TTX was found in the skin, viscera, and muscle of a goby (*Y. criniger*), inhabiting the Amami and the Ryukyu Islands, Japan. This goby is also distributed in the Philippines and Taiwan, where it has been implicated in several human poisoning cases. In addition, TTX was detected in the skin of *Atelopus* frogs inhabiting Costa Rica and Panama.

In 1978, TTX was isolated from the posterior salivary gland of the blue-ringed octopus (*O. maculosus*), which mainly inhabits northern Australia. Human TTX cases are occasionally reported in this area due to envenomation by *O. maculosus*. This octopus also occasionally appears in middle to south Japan. The octopus secretes TTX from the posterior salivary gland to paralyze its prey.

In 1979, a serious poisoning incident due to ingestion of the trumpet shell (*C. sauliae*), which is widely consumed, occurred in Shimizu, Shizuoka, Japan. The causative agent was isolated from the digestive gland and identified as TTX (Narita *et al.*, 1981). Two similar food poisoning incidents followed in Wakayama and in Miyazaki, Japan, in 1982 and 1987, respectively. The toxic carnivorous gastropods are as follows: the frog shell or "oonarutobora" (*T. lissostoma*), "hanamushirogai" (*Zeuxis siquijorensis*), and "araregai" (*N. clathrata*) in Japan and *N. lineata* (Hwang *et al.*, 1991a,b) in Taiwan also contain TTX in their digestive glands. In addition, in 1980, TTX was detected in the ivory shell (*B. japonica*), collected in Fukui, Japan.

Mass food poisonings due to ingestion of the eggs of the horseshoe crab sporadically occur in Thailand (Kanchanapongkul and Krittaya, 1995), where during early 1996 they caused about 20 deaths. TTX in the eggs was later determined to be the cause of death. Xanthid crabs (*A. floridus, Z. aeneus*), flatworms (*Planocera* spp.), and ribbon worms (Nemertinea) have also been shown to possess TTX.

Presently, distribution of TTX is known in only a limited number of organisms (Table II). Toxicity data on TTX-bearing animals collected show more or less toxicity in them irrespective of local and individual variations of toxicity. All of them are found to be carnivorous. TTX in them seems to come directly from their food, such as in puffers, toxic gastropods, flatworms, and starfish, and is accumulated in species-specific organ(s).

Since TTX is distributed in a variety of invertebrates and vertebrates and there is a wide individual variation of toxin content, even among members of the same species, the origin of TTX was deduced to be a universal organism such as a microbe. The reason why TTX-bearing animals struggle to accumulate TTX may be different dependent on species. However, in the case of puffers, newts, flatworms, and horseshoe crabs, TTX may play a defensive role against predators, especially to prevent their eggs from the enemies. In other animals, TTX may be used as the offensive substance to immobilize and capture prey by secretion or envenomation of the toxin from the proboscis in ribbon worms or salivary glands in the blue-ringed octopus.

Using instrumental HPLC and GC–MS analyses for TTX, an intestinal bacteria isolated from a toxic xanthid crab (*A. floridus*) collected from Shimoda, Japan, was discovered to produce TTX (Noguchi *et al.*, 1986b). This bacterium was also noted as analogous to *Vibrio fischeri*. Soon after, *V. alginolyticus* and several other *Vibrio* spp. isolated from the intestines of the puffer *T. snyderi*, the starfish *A. polyacanthus*, the blue-ringed octopus *O. maculosus*, and the horseshoe crab *C. rotundicauda* were also demonstrated to produce TTX using similar analytical techniques. Simidu *et al.* (1987) showed that 12 of 24 type culture strains of marine bacteria tested clearly produced TTX or related substances (Table XV). Yasumoto *et al.* (1989) and Matsui *et al.* (1990) ascertained that *A. tetraodonis* isolated from a calcareous alga (*Jania* sp.) and *S. putrefaciens* isolated from a puffer (*T. niphobles*) produced TTX and anhydroTTX, respectively. Typical TTX production by *Vibrio* group VIII (Hashimoto *et al.*, 1990) isolated from the intestines of *A. floridus* was demonstrated through instrumental analyses for TTX as well as cell toxicity assays. An appreciable amount of TTX (30 MU/flask) was produced by *Vibrio* group VIII strain (Table XVI).

TABLE XVI

ANALYSIS FOR TTX AND RELATED SUBSTANCES IN THE EXTRACTS OF SEVERAL *VIBRIO* GROUPS OF BACTERIA ISOLATED FROM INTESTINES OF *ATERGATIS FLORIDUS* SPECIMENS COLLECTED FROM KOJIMA IN ISHIGAKI ISLAND, OKINAWA

		HPLC		
Vibrio group	Lethal potency (MU)	TTX	AnhydroTTX	GC–MS
I	–	+	–	
III	–	+	–	
V	–	±	–	
VIII	30	+	+	+

Source: Hashimoto *et al.* (1990).

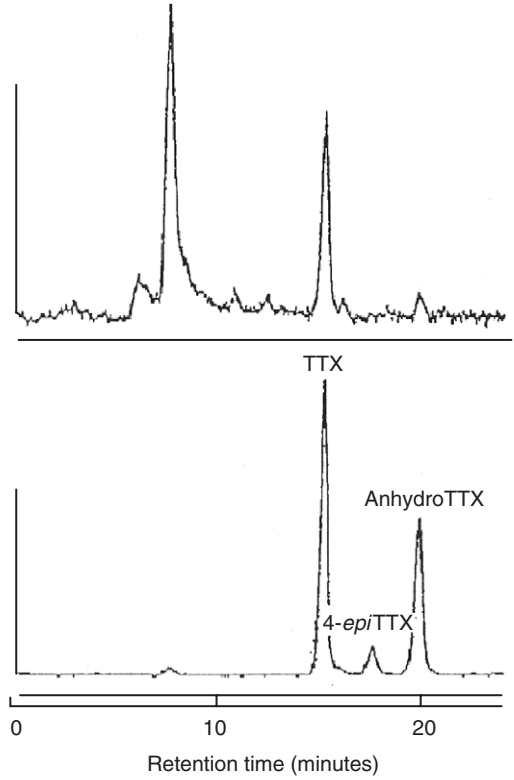


FIG. 5 HPLC of TTX fraction forms *Vibrio* group VIII isolated from a Kojima specimen (upper) and of authentic TTX (lower) (Noguchi *et al.*, 1986b).

The TTX fraction showed two peaks (TTX and anhydroTTX) in HPLC analysis, and in GC–MS analysis for the toxin (C_9-base) exhibited mass fragment ions at *m/z* 407 (molecular peak), 392 (base peak), and 376, all of which are specific to trimethylsilylated C_9-base derived from authentic TTX (Figures 5 and 6). It is recognized from the above evidence that many bacteria can produce TTX and/or its derivatives. TTX productivity *in vitro*, however, seems largely dependent on culture conditions. The optimal culture conditions for maximum toxin yield by TTX-producing bacteria remain unknown. Such bacteria may produce minimal amounts of TTX in the intestines of fish and invertebrates. In addition, on the basis of the following observations it is presumed that the carnivorous TTX-bearing organisms are able to accumulate the toxin from TTX-producing bacteria:

- Trumpet shells can become highly intoxicated by ingesting toxic starfish (Noguchi *et al.*, 1982).
- The debris of small gastropods is often found in the digestive ducts of toxic puffers (Jeon *et al.*, 1984).
- Cultured puffers (nontoxic) can become intoxicated by feeding on the liver of toxic puffers and do not become intoxicated by feeding on a nontoxic diet in net cages (Noguchi *et al.*, 2004).
- The starfish accumulated a lot of TTX from small toxic gastropods (Lin and Hwang, 2001).
- Cultured puffers were intoxicated when the toxic flatworm appeared in the aquaculture pond (Lin *et al.*, 1998a).

However, during elucidation of several food poisoning cases due to ingestion of TTX-bearing organisms, TTX intoxication mechanism of causative organisms was found to come from their food chains. They were confirmed from their designed model and were reasonably proved to come from their TTX-containing diet. There have been a few attractive documents in favor of the exogenous

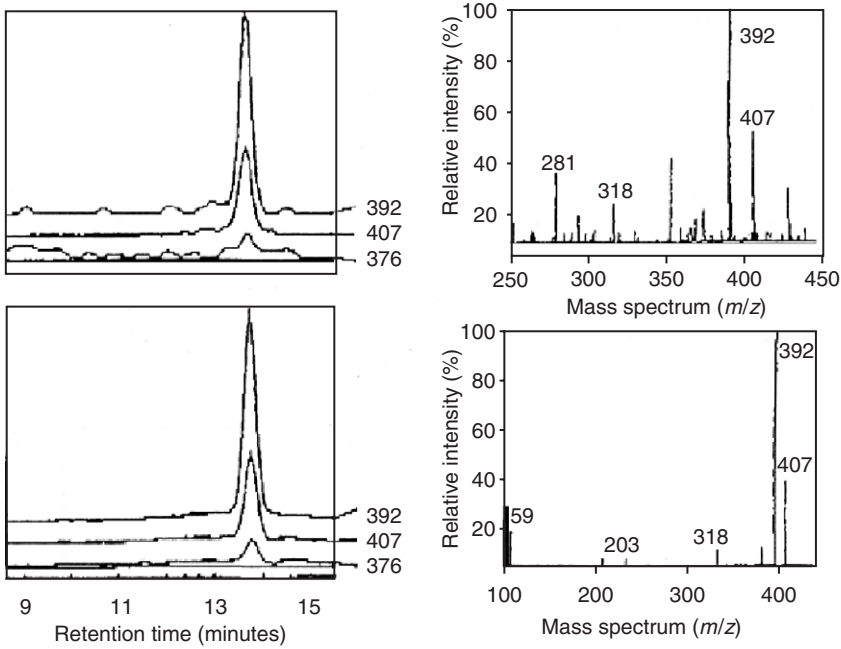


FIG. 6 GC–MS of TTX fraction of *Vibrio* group VIII from a Kojima specimen. GC, left; MS, right (Noguchi *et al.*, 1986b).

origin of TTX. Meanwhile, several attempts were made to confirm the exogenous speculation and it was postulated that bacteria are responsible for the production of TTX (Noguchi *et al.*, 1986b, 1987; Yasumoto *et al.*, 1986). In support of this postulation, there are two possible TTX intoxication routes described so far. First, some bacteria produce TTX in the sediment and then the toxin is transferred to higher animals through the food web. Second, the host animals accumulate TTX through the symbiotic way. These ideas have been generated and justified from the discovery of TTX-producing bacteria in various toxic host animals and in marine sediments as well, as described in the previous section.

TTX is one of the best-known notorious marine toxins that occasionally cause human intoxication, including fatality. Poisoning incidents owing to TTX have been almost exclusively associated with ingestion of toxic puffer and gastropod, especially from the waters of the Indo-Pacific Ocean regions.

The toxicosis is characterized by the onset of symptoms of the victim. The treatment of the illness is mainly based on the symptoms of the patient. More fruitful treatment can be provided if the causative toxin is identified. Detection and determination of TTX are therefore essential not only for diagnosis and treatment purposes, but also for making quarantine rule and public awareness. Quantitative and/or qualitative detection of TTX in a sample are/is performed by several chemical and biological methods, as described below.

D. TTX DETECTION METHOD

1. Mouse bioassay

The features of detection methods for TTX are shown in Table XVII. Some methods including bioassay, HPLC, and liquid chromatography–mass spectrometry (LC–MS) are usually used to qualitatively and quantitatively detect TTX, but other methods including GC–MS, IR, and NMR are usually used to qualitatively detect TTX. Among them, LC–MS is the most powerful and sensitive tool for qualitatively and quantitatively determining TTX.

Mice have been commonly used for determination of toxicity for TTX. To know the concentration of TTX in a sample of interest, the mouse bioassay is used and described above.

Mouse bioassay is also used to identify an unknown toxin extract in comparison with a TTX-specific dose–death time relationship curve. A series of test solutions are prepared by diluting the unknown toxin extract with 0.1% acetic acid. Aliquots of each test solution are intraperitoneally injected into a group of mice. Using the median value of their death time at each dilution level, the dose–death time curve is drawn, which provides the nature

TABLE XVII

THE FEATURES OF DETECTION METHODS FOR TTX

Method	Detection system	Detection limit	Features	References
Bioassay	Mice	0.2-μg TTX	Death time	Hwang and Jeng, 1991; MHW, 1991
HPLC	Fluoromonitor	0.03-μg TTX	Intensity	Nagashima *et al.*, 1987; Yasumoto *et al.*, 1982
TLC	Fluorescent spot	2-μg TTX	Spot mobility	
Electrophoresis	Fluorescent spot	2-μg TTX	Spot mobility	
Capillary isotachophoresis	Potential stand	0.25-μg TTX	Ion mobility	Shimada *et al.*, 1983
UV spectroscopy	Spectrometer	Qualitative	Alkali decompose	Suenaga and Kotoku, 1980
GC–MS	Mass spectrometer	Qualitative	Alkali decompose + ion-monitoring	Narita *et al.*, 1981
IR spectrometry	IR spectrometer	Qualitative	Functional group	Onoue *et al.*, 1984
FABMS	Mass spectrometer	Qualitative	Ion-monitoring	Noguchi *et al.*, 1991
LC–MS	Mass spectrometer	0.05-ng TTX	Ion-monitoring	Hwang *et al.*, 2005; Tanu and Noguchi, 1999
ESI-TOF/MS	Mass spectrometer	Qualitative	Ion-monitoring	Tanu *et al.*, 2001
NMR spectrometry	NMR spectrometer	Qualitative	Proton spectrum	Nakamura and Yasumoto, 1985; Tsuruda *et al.*, 2001; Yotsu-Yamashita, 2001
Cytotoxicity test	Microscopic examination	3 nmol/liter	Cell death	Hamasaki *et al.*, 1996; Kogure *et al.*, 1988
Immunoassay	ELISA	2 ng/ml	Monoclonal antibody	Matsumura and Fukiya, 1992; Watabe *et al.*, 1989

of the toxin since the dose–death time curve is specific to toxin. For example, Hashimoto and Noguchi (1971) preliminarily identified goby toxin as TTX with considerable accuracy, using the dose–death time curve (Figure 7). Hwang *et al.* (1990a) also identified gastropod toxin as TTX using the dose–death time curve.

Although the mouse has been the animal of choice for determination of TTX, it is not without controversy due to some potential drawbacks related to the bioassay, such as low accuracy caused by the individual variation inherent to biological system, lack of specificity, inconvenience of purchasing a particular size of mice of specific strain, and recent international movement of prevention from cruelty to animals. These limitations have spawned the development of several alternative chemical methods, with the goal of obtaining comprehensive tests surpassing the mouse assay, as illustrated under the following sections (Noguchi and Mahmud, 2001).

2. *High-performance liquid chromatography*

HPLC methods have been explored for both qualitative and quantitative analyses of TTX and its analogues by many researchers. The fluorometric HPLC method is mainly composed of a high-pressure piston pump with a

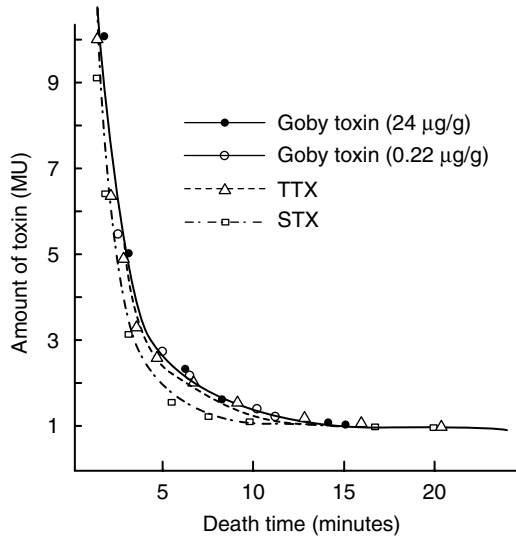


FIG. 7 Dose–death times curve for goby toxin, TTX, and saxitoxin (STX) (Hashimoto and Noguchi, 1971).

syringe loading sample injector, stainless column, reaction pump for delivering reaction reagent, fluoromonitor, and chromatorecorder for calculation of peak area. Toxin is first separated from the contaminants with a buffer system on a column packed with either silica gel C-18 or ion exchange resin, and the toxin eluate is then mixed with NaOH. The toxin is converted into fluorescent compounds and then passed through a tube placed in an aluminum block oven. Eventually, when the fluorescent compounds are passed through a fluoromonitor equipped with a lamp, their retention times of the toxin and fluorescence intensities are recorded, showing chromatogram in the chromatorecorder. Toxins are identified from the retention times of the authentic TTXs. In quantitative analysis of HPLC, the detection limit of the authentic TTX is about 0.03 μg. Until now, several attempts have been made to detect TTX and its analogues under different conditions of HPLC, and a number of advances in our understanding of the biochemistry of TTXs are a direct result of these developments. Briefly, some promising methodologies can be described here.

In early 1980s, a fluorometric continuous TTX analyzer was constructed (Yasumoto *et al.*, 1982). In this system, the toxin was first separated from contaminations on a column of a weak cation exchange gel (Hitachi Gel 3011C, Tokyo) with a 0.06 mol/liter citrate buffer solution (pH 4.0), and toxin concentrations of above 8 MU/g were detected. Due to the poor performance in TTX analogues, improved analyzer was constructed later by Yotsu *et al.* (1989). The improved method could detect TTX derivatives including 6-*epi*TTX, 4,9-anhydro-6-*epi*TTX, 4-*epi*-11-*deoxy*TTX, 11-*nor*TTX-6(*R*)-ol, 4-*epi*TTX, 4,9-anhydroTTX, 11-*deoxy*TTX, and 4,9-anhydro-11-*deoxy*TTX isolated from puffer and newt specimens. TTX derivatives were separated on a Develosil column (1.0×25 cm^2) with 0.06 mol/liter heptafluorobutyric acid in 0.001 mol/liter ammonium acetate buffer (pH 5.0). An amount of 4 mol/liter NaOH was used to produce fluorescent compounds. Separation of TTX from 6-*epi*TTX is, however, considered as the major achievement by this improved analyzer. This analyzer is especially useful for monitoring tropical animals, which contain a considerable amount of 6-*epi*TTX. Reversed-phase ion-pairing HPLC method has also been the system of choice by many researchers for the fastest and efficient analysis of TTX and its analogues, where heptanesulfonic acid (HAS) is used as counterion (Nagashima *et al.*, 1987). In this method, the detection reagent for TTX and related substances does not react with any PSP component if present in the contaminant sample. Table XVIII represents a reversed-phase HPLC condition according to the method of Nagashima *et al.* with slight modification (Arakawa *et al.*, 1994), being used to analyze partially purified Japanese newt (*C. pyrrhogaster*) poison, TTXs (Figure 8) (Tsuruda *et al.*, 2002).

TABLE XVIII
OPERATING CONDITIONS OF HPLC FOR THE ANALYSIS OF NEWT TOXINS TTXs

HPLC system	D-7480 Hitachi
Column (4.6 × 250 mm^2, GL Sci., Inc., Japan)	Inertsil ODS-3
Column temperature	30°C
Mobile phase	60-mM ammonium phosphate buffer (pH 5.0) containing 10-mM HAS and 2% acetonitrile
Reagent	4-M NaOH
Flow rate	0.8 ml/minute
Reaction temperature	110°C
Detection	Excitation 384 nm, emission 505 nm

Source: Tsuruda *et al*. (2002).

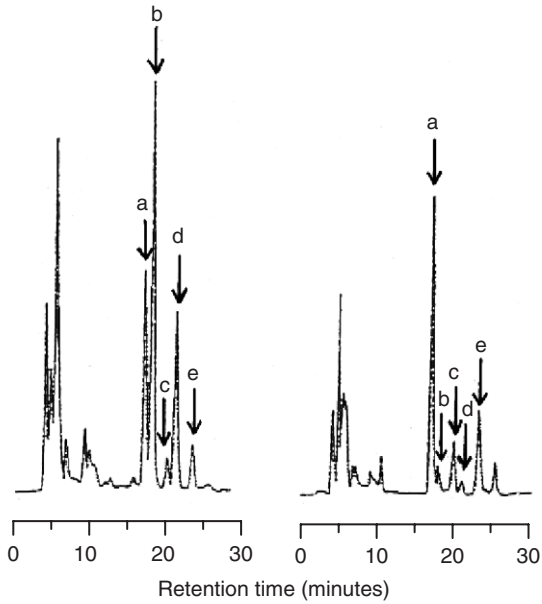


FIG. 8 HPLC of authentic TTXs (left) and the Japanese newt (*C. pyrrhogaster*) toxin (right). a, TTX; b, 6-*epi*TTX; c, 4-*epi*TTX; d, 4,9-anhydro-6-*epi*TTX; e, 4,9-anhydroTTX (Tsuruda *et al*., 2002).

3. *Thin-layer chromatography*

In TLC analysis, TTX is spotted onto a silica gel-60 F_{254} precoated plate (Merck, Darmstadt). The plate is developed in three different solvent systems of pyridine–ethyl acetate–acetic acid–water (15:5:3:4), 3-butanol–acetic acid–water (2:1:1), and 1-butanol–acetic acid–water (12:3:5) solvent in a sealed container. The solvent rises by capillary action and an ascending chromatographic separation is obtained. The plate is then sprayed with 10% KOH followed by heating at 100°C for 10 minutes. The toxin is visualized as a yellow fluorescent spot under UV light (365 nm). In TLC analysis, the R_f values of TTX are around 0.70, 0.45, and 0.20 with pyridine–ethyl acetate–acetic acid–water, 3-butanol–acetic acid–water, and 1-butanol–acetic acid–water solvent, respectively. It is also possible to detect TTX on the TLC plate using the Weber reagent that gives pink spot of the toxin. In TLC, the detection limit is about 2 μg of TTX (10 MU). TLC is a useful technique in those laboratories where HPLC and other costly analytical systems are not available.

4. *Electrophoresis*

Electrophoresis is a relatively simple and rapid method with high resolution detection of polar compounds like TTX. When 1 μl of TTX (10 MU, corresponding to 2 μg) is applied onto a 5 × 18 cm^2 cellulose acetate membrane (Chemetron, Milano), the ion molecules of TTX move toward the cathode with a mobility (Rm) clearly smaller than that of authentic STX. The analysis is performed for 30 minutes in an electrolytic buffer solution of 0.08 mol/liter Tris-HCl (pH 8.7), under the influence of an applied electric field with a constant current of 0.8 mA/cm width. The toxin is visualized in the same manner as described for TLC.

5. *Capillary isotachophoresis*

Capillary isotachophoresis is a rapid, accurate, and potential detection technique for TTX. A small amount of TTX in contaminated extracts can be determined by this method (Shimada *et al.*, 1983). It is performed using a cationic system, as TTX exists as cation under acidic and neutral conditions. Conditions for capillary isotachophoresis composed of 5 mmol/liter potassium acetate (pH 6.0) as an electrolyte, containing 0.2% Triton X-100 and 0.5 volume of dioxane, and 10 mmol β-alanine adjusted to pH 4.5 with acetic acid as a terminating electrolyte. When TTX is applied to isotachophoretic analyzer (Shimadzu IR-2A) equipped with a potential gradient 0.32, it is eventually monitored by the detector. PU is expressed as (PG_S-PG_L), where PG_S, PG_L, and PG_T stand for potential gradient values for sample, leading ion, and

terminating ion, respectively (Miyazaki and Katoh, 1976). The quantitative detection limit by this method is about 0.25 μg of TTX (1.2 MU).

6. *UV spectroscopy*

In UV spectroscopy, TTX is generally determined by irradiating a crude toxin with UV light. A small amount of TTX is dissolved in 2 ml of 2 mol/liter NaOH and heated in a boiling water bath for 45 minutes. After cooling at room temperature, the solution is examined for the UV absorption spectrum, characteristic to the C_9-base, 2-amino-6-hydroxymethyl-8-hydroxyquinazoline which should have been derived from TTX and/or related substances, if present (Suenaga and Kotoku, 1980). In the analysis, UV spectrum of the alkali-decomposed compounds of TTX appears as a shoulder at around 276 nm (Tanu and Noguchi, 1999), indicating the formation of the C_9-base (Figure 9), specific to TTX or related substances.

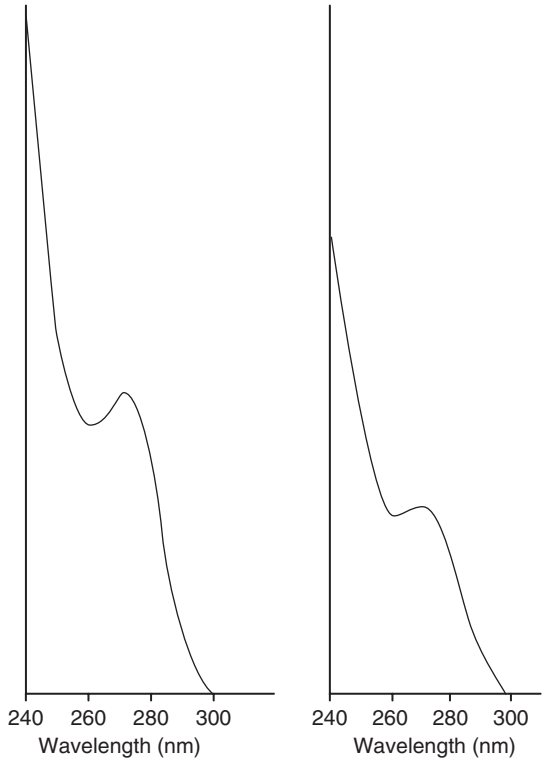


FIG. 9 UV absorption spectra of alkaline hydrolyzates of authentic TTX (left) and horseshoe crab (*C. rotundicauda*) toxin (right) (Tanu and Noguchi, 1999).

7. *Gas chromatography–mass spectrometry*

GC–MS analysis is an indirect method to detect TTX in a crude extract which is difficult to purify for other advanced analysis. In this method, TTX and its derivatives (0.2 ml with 25 MU) are dissolved in 2 ml of 2 mol/liter NaOH and heated in a boiling water bath for 45 minutes. After being cooled at room temperature, the alkali-decomposed compounds are adjusted to pH 4.0 with 1 mol/liter HCl and extracted thrice with three volumes of 1-butanol. The extracts are combined and evaporated to dryness in vacuum, and to the residue is added a mixture of *N,O-bis* acetamide, trimethylchlorosilane, and pyridine (2:1:1), in order to derive trimethylsilyl (TMS) "C_9-base" compounds. The derivatives are then submitted to GC–MS analysis. The column temperature is maintained from 180 to 250°C at a rate of 5°C/minute. The flow rate of inlet helium carrier gas is maintained at 20 ml/minute. The ionizing voltage is usually kept at 70 eV with the ion source temperature at 200°C.

An example of MS of the selected ion-monitored chromatograms (SIM) from the TMS derivatives of alkali-decomposed puffer *T. oblongus* poison TTX ("C_9-base") is shown in Figure 10. Sharp fragment ions appear at *m/z* 407 (parent peak), 392 (base peak), and 376, indicating the presence of the quinazoline skeleton in the toxin (Narita *et al.*, 1981).

8. *IR spectrometry*

IR spectrometry is the analytical technique for determination of functional groups of TTX. IR spectra of TTX are measured in a KBr pellet, using a JASCO model IR-S spectrophotometer (Tokyo) (Onoue *et al.*, 1984). Figure 11 represents an IR spectrum of puffer toxin, showing characteristic absorption in 3360 (OH), 3200, 1660 (guanidium), 1610 (COO^-), and 1075 cm^{-1}.

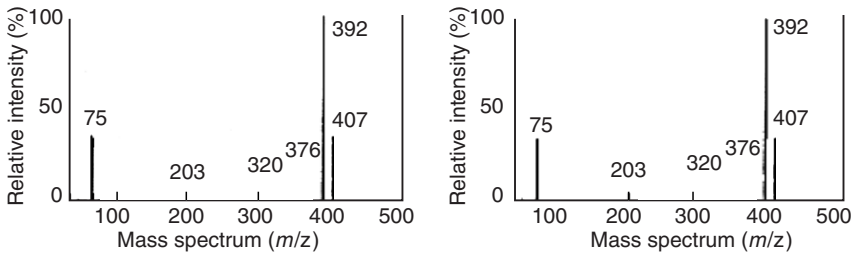


FIG. 10 Mass spectra of C_9-base-$(TMS)_3$ derivative from a marine puffer toxin (left) and authentic TTX (right) (Narita *et al.*, 1981).

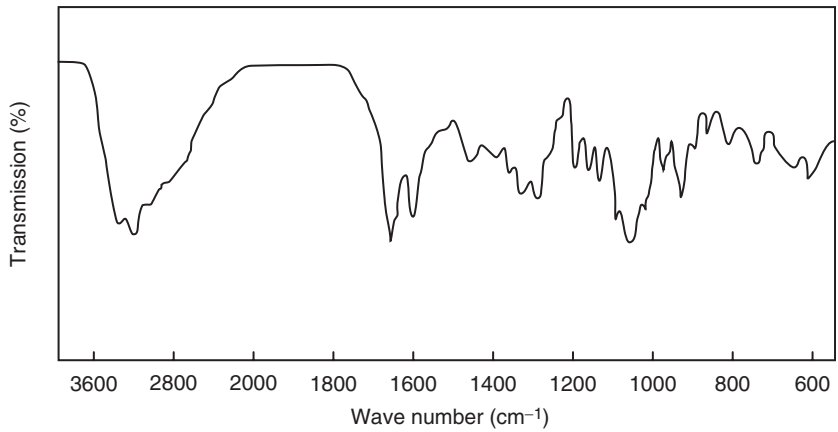


FIG. 11 IR spectrum of puffer toxin (Onoue *et al.*, 1984).

Although the spectrum in IR analysis is presumed to be complex, it is a helpful tool for identification of TTX.

9. *Fast atom bombardment mass spectrometry*

FABMS is a direct method for the qualitative confirmation of TTX. The analysis is carried out on a JEOL JMS DX-300 mass spectrometer (Tokyo) equipped with a JEOL DA-5000 data system (Noguchi *et al.*, 1991). Xenon is used to provide the primary beam of atoms, the acceleration voltage of the primary ion being 3 kV. Scanning is repeated within a mass range of *m/z* 100–500. In this analysis, about 0.1 mg of TTX and glycerol as matrix are placed on the sample stage of the mass spectrometer, mixed well, and submitted to the ion chamber of the spectrometer (FAB). Both positive and negative mass spectra of TTX are then measured. As shown in Figure 12, TTX shows $(M + H)^+$ and $(M + H - H_2O)^+$ ion peaks at *m/z* 320 and 302, respectively, in the positive mass spectrum, and an $(M - H)^-$ peak at *m/z* 318 in the negative spectrum. Secondary ion MS, performed by a Hitachi M-80B mass spectrometer equipped with a Hitachi M-0101 data system, presented essentially the same result as shown by FABMS. Extensively purified sample is required for the successful application of this method.

10. *Liquid chromatography–mass spectrometry*

LC–MS is developed for the detection of TTX with considerable accuracy (Shida *et al.*, 1998). In this method, combined HPLC–MS is performed using Hitachi M-1000 system coupled to a mass spectrometer. HPLC system is

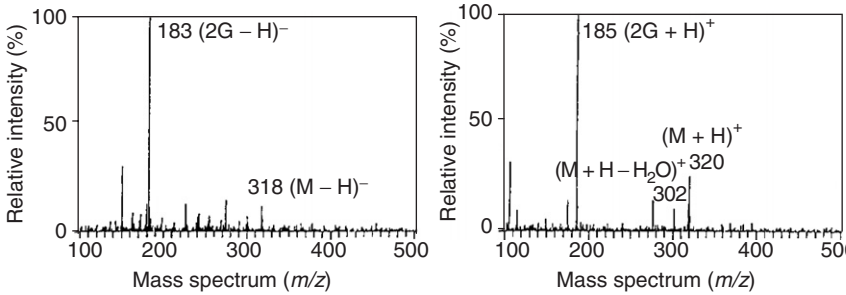


FIG. 12 Positive (right) and negative (left) FAB mass spectra of TTX (Noguchi *et al.*, 1991).

equipped with an ODS-3 (1.5 × 150 mm) column. Acetonitrile (50%, flow rate 70 μl/minute) is used as mobile solvent. The effluent from the column is split to provide flow to the ion-spray interface.

An example of mass spectra of a brackish water puffer toxin in LC–MS analysis is shown in Figure 13 (Tanu and Noguchi, 1999). In the MS, a protonated molecular ion peak $(M + H)^+$ appeared at $m/z = 320$ showing the molecular weight of the toxin of 319, in good accordance with that of TTX.

Recently, we found that the combination of C-18 Sep-Pack cartridge and Ultrafree micro centrifuge filters with LC–MS (California) is very useful in detecting TTX from the urine and blood samples for poisoned patients for diagnosis of TTX food poisoning. The detection limit of TTX in biological sample was 12.5 nM (equivalent to about 3.9 ng/ml or 0.02 MU/ml) by LC–MS (Hwang *et al.*, 2005).

11. Electrospray ionization-time of flight/mass spectrometry

ESI-TOF/MS is a valuable technique for determination of TTX, although it is not widely used so far in marine toxin determination. In this analysis, a portion of purified TTX (less than 0.05 mg) is dissolved in a small amount of 1% acetic acid, and added to 50% aqueous methanol. ESI-TOF/MS is taken on a Micromass Q-Tof Mass Spectrometer (Tokyo). Recently, TTX in a tree frog *Polypedates* sp. extract has been successfully analyzed by ESI-TOF/MS analysis (Tanu *et al.*, 2001). As shown in Figure 14, in the spectrum of the toxin, protonated molecular ion peak $(M + H)^+$ appeared at $m/z = 320.1103$, suggesting the molecular weight of the toxin to be 319.1025 which agrees well with that of authentic TTX ($C_{11}H_{17}N_3O_8 = 319.1016$).

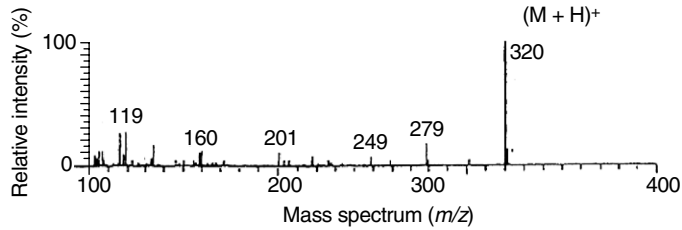


FIG. 13 Mass spectrum of chromatogram at 8.6 minutes from a brackish water puffer (*T. nigroviridis*) toxin in LC–MS analysis (Tanu and Noguchi, 1999).

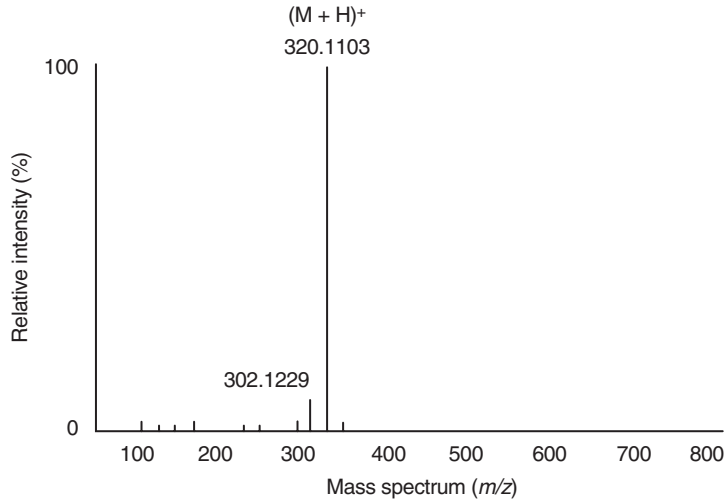


FIG. 14 ESI-TOF/MS analysis of a frog (*Polypedates* sp.) toxin: *y*-axis, relative intensity; *x*-axis, mass spectrum at *m/z* (Tanu *et al.*, 2001).

12. ^{1}H NMR spectrometry

^{1}H NMR has been playing an important role as a complementary method to determine the absolute configuration of TTX and its derivatives. At present 13 derivatives of TTX have been isolated, whose ^{1}H NMR data are presented by various investigators (Endo *et al.*, 1988; Nakamura and Yasumoto, 1985; Tsuruda *et al.*, 2001; Yasumoto *et al.*, 1988; Yotsu *et al.*, 1990a,b, 1992a,b,c; Yotsu-Yamashita, 2001).

In ^{1}H NMR analysis, the lyophilized powder of purified toxin is dissolved in 0.6 ml of 4% acetic-D_3 acid-D (99.5% purity) in D_2O and placed in an

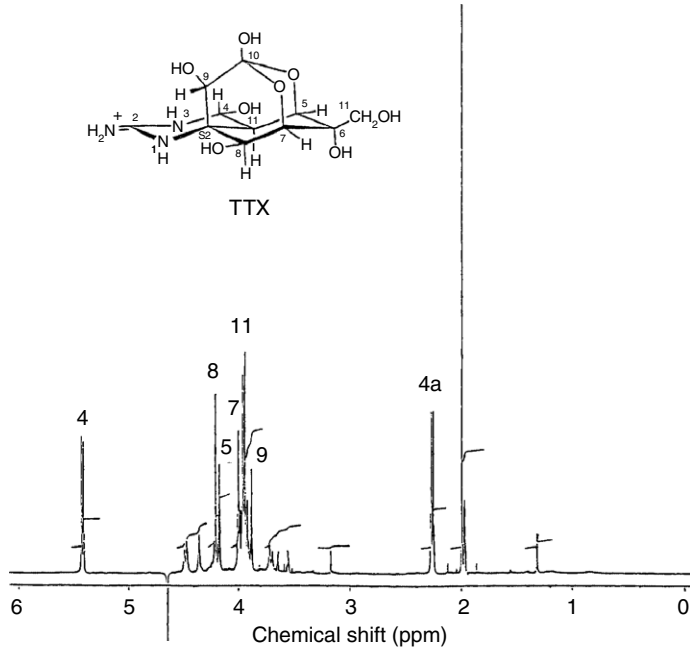


FIG. 15 ^{1}H NMR spectrum of a brackish water puffer (*T. nigroviridis*) toxin (Mahmud *et al.*, 1999a).

NMR tube. Tetramethylsilane (Me_4Si) is used as an external standard. The spectrum is taken on a Varian Unity Plus 500 spectrometer at 500 MHz. Figure 15 exhibits signals at 2.03 (s, CH_3COOD), 2.34 (d, J = 9.6 Hz), 3.97 (s), 4.05 (s), 4.26 (s), 4.29 (s) and 5.44 (d, J = 9.4 Hz) ppm. C4-H was coupled with C4a-H with a spin-spin-coupling constant of almost same of 9.4 and 9.6, respectively, which is a typical characteristic of TTX.

13. Cytotoxicity test

Cell bioassays are effective biological screening tools for a wide range of drugs, chemicals, and toxic compounds. The utility and design of these *in vitro* methods are described by the mechanism of action associated with the compounds of interest and the availability of appropriate target cell lines. For the quantitative measurement of a sodium channel blocker TTX, even at low level of detection (~3 nmol/liter) in marine bacteria, the tissue culture bioassay (TCBA) has been used (Kogure *et al.*, 1988). This assay is based on the ability of sodium channel-blocking toxins to antagonize the

combined effects of two chemicals, veratridine and ouabain, on neuroblastoma cell lines (ATCC, CCL-131). Veratridine (0.075 mmol/liter) and ouabain (1 mmol/liter) cause the cells to round up and die subsequently. Cells continue to grow in the presence of TTX, as TTX counteracts the veratridine. The amount of toxin is estimated from the linear relationship of the relative abundance of living cells and the concentration of toxin in the samples. The results are scored by visually noting the morphology of a significant number of cells under microscope. The sensitivity of this method is much higher than that of the mouse assay. This method is, however, not always convenient for routine bioassays, because it is time consuming, and needs some experience to distinguish dead cells from living cells during microscopic examination.

To replace the time-consuming and subjective cell-counting procedure, TCBA in combination with a water-soluble tetrazolium salt 2-(4-iodophenyl)-3-(4-nitriphenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was improved for the quantitative measurement of TTX, using a microplate reader (Kogure *et al.*, 1988). WST-1 is used as an indicator of living cells. TTX concentrations can be measured between the range of 2 and 70 nmol/liter by this method. WST-1 TCBA is also a useful assay for the detection of TTX in a supernatant of bacterial culture and its sensitivity is several times higher than that of TCBA as reported by Hamasaki *et al.* (1996). This method seems to be promising, owing to the omission of both washing and dissolving steps, which allows greater reproducibility of the assay.

14. Immunoassay

Immunoassays are commonly used as inexpensive, sensitive, and highly selective methods for the detection and quantification of a wide variety of drugs and other substances of biomedical significance (Raybould *et al.*, 1992). For TTX detection, several immunoassay techniques have been developed so far, without much success. Watabe *et al.* (1989) developed an enzyme-linked immunosorbent assay (ELISA) system by raising a monoclonal antibody (Mab) against a TTX derivative TDA. In this approach, TDA was conjugated with bovine serum albumin (BSA) and injected intraperitoneally into Balb/c mice. Spleen cells of mice were then isolated and fused with myeloma cells X63-Ag8-6.5.3, and cloned. Mab was produced in ascites fluid in the mouse by cloned cell and its detection capability for TTX was 0.03–100 μg/well (0.3–1000 μg/ml). This method had, however, low sensitivity. Huot *et al.* (1989) reported the production of two anti-TTX Mabs. Binding of these antibodies was only partially inhibited (48% and 25%) by free TTX at a concentration of 50 ng/ml, showing, however, a low performance. Raybould *et al.* (1992) developed a rapid, significantly sensitive and specific competitive inhibition enzyme immunoassays (CIEIAs) for TTX

detection and quantification in biological samples, by using a newly developed Mab from mice. In this CIEIA approach, Mab detected TTX with sensitivities at IC_{50} and IC_{20} of 6–7 ng/ml, respectively. Other group reported the competitive enzyme-linked immunoassay (EIA) for TTX determination, although these methods required a long time for the analysis (Matsumura, 1995a,b; Matsumura and Fukiya, 1992). Recently, a Mab against TTX has been developed from Balb/c mice immunized with TTX–BSA conjugate, by which a rapid (generally takes 30 minutes) and highly sensitive EIA system has been established for quantitative analysis of TTX. In this promising method, Mab can detect TTX at concentrations of 2–100 ng/ml. This method is considered as a useful tool for monitoring TTX in puffer fish or other seafoods. Using this highly specific Mab, a useful immunoaffinity column chromatography has also been developed for isolation and identification of TTX from the urine samples of a poisoned patient (Kawatsu *et al.*, 1997, 1999). This chromatographic technique is performed in combination with fluorometric HPLC. The maximum recovery rate of TTX by this immunoaffinity is reported to be 88%. The detection limit of TTX is 2 ng/ml of the urine. For diagnosis of TTX poisoning patients, this chromatographic method might be used as a potential tool.

E. CHEMISTRY OF PUFFER TOXIN

Puffer toxin generally distributes toxic puffers. Japanese people have a habit to eat puffers' muscle and liver for a long time and, accordingly, have encountered food poisoning, sometimes resulting in death. On account of many and serious poisonings due to ingestion of puffer in Japan, many Japanese scientists have, so far, studied on its chemical and pharmacological characterizations for a long time. Dr. R. Tahara succeeded in partial purification of the puffer toxin in 1909 and named it as TTX after its scientific name. The absolute structure of TTX was elucidated by three groups of scientists: Goto *et al.* (1965), Tsuda *et al.* (1964), and Woodward (1964) in 1964, and the synthesis of racemic TTX was further reported by Kishi *et al.* (1972). In 1999, synthesis of (–)-5,11-*dideoxy*TTX (Yotsu-Yamashita *et al.*, 1999), together with its 4-*epi*TTX (Yasumoto and Michishita, 1985) and 4,9-anhydroTTX (Yotsu *et al.*, 1989) analogues, was achieved by Nishikawa *et al.* (1999) as the first stereocontrolled total synthesis of TTX analogues. The TTX origin deriving from bacteria was also reported by two groups. Since then, 4-*epi*TTX (Tsuda *et al.*, 1964), 4,9-anhydroTTX (anhydro-*epi*TTX or anhydroTTX) (Goto *et al.*, 1965), and TDA (Woodward, 1964) were isolated from the puffer as minor components. In the acidic aqua, 4-*epi*TTX and 4,9-anhydroTTX are in equilibrium with TTX. Crystalline TTX forms hemilactal type (Woodward, 1964) while in the acidic aqua, TTX forms protoned hemilactal type with bilateral ions where the hemilactal equilibrates

with the hydroxy lactone (Woodward, 1964). Other TTX derivatives have already been isolated and characterized as shown in Figure 2.

Pure TTX is insoluble in water and organic solvents. However, in strongly acidic aqua it is soluble and stable while in alkaline one it is unstable. Molecular weight is 319. It does not show clear melting point and beyond 200°C, it changes into black color. TTX has a guanidium function in its molecule. Specific toxicity of TTX is 5000–8000 MU/mg where 1 MU is defined as amount of TTX that can kill a male ddY strain mouse of body weight 20 g in 30 minutes, corresponding to about 0.2 μg of authentic TTX. MLD_{50} for human is about 2 mg. This toxicity is almost equivalent to that of STX, a typical component of PSP which is one of the most potent neurotoxins among toxins with low molecular weight. Monoclonal TTX antibody to detect TTX with high sensitivity has been developed by two groups (Kawatsu *et al.*, 1997, 1999) and consequently, microdistribution of TTX in several tissues of the puffer (Mahmud *et al.*, 2003a,b; Tanu *et al.*, 2002) and the newt (Tsuruda *et al.*, 2002) was elucidated. However, antibody to cure TTX-poisoning patients remains to be developed.

TTX is supposed to bind with some high molecular weight substance such as protein or to occur as water-soluble precursor(s) instead of free TTX since free TTX is water insoluble and if alone, TTX cannot distribute the cell of TTX-bearing organisms.

F. PHARMACOLOGY OF TTX

1. Introduction

TTX is a potent neurotoxin originally found in the ovary and liver of puffer, followed by some tissue(s) in TTX-bearing animals such as newts, gobies, frogs, gastropods, and so on. From the recent investigations, it has now become abundantly clear that origin of TTX is exogenous from a food web in the puffer. The pharmacology of TTX had been studied for a long time especially in Japan to prevent food poisoning since puffers are regarded as the most delicious fish among Japanese. On the basis of previous literatures, TTX is well known to play the selective and potent blocking action on the sodium channel (Ebesu *et al.*, 2000; Narahashi, 2001; Narahashi *et al.*, 1960). Recently, its cellular and molecular mechanism of action has been extensively studied. The fact is equally important that TTX has since then been used extensively as a chemical tool in the laboratory for the purpose for studying the sodium channel, other ion channels, and various aspects of membrane excitability and synaptic transmission.

On the basis of the achievements of recent pharmacological studies for TTX, TTX is suggested to play a role of defense and offense against enemy

TABLE XIX
TTX RESISTIBILITY IN TTX- AND NON-TTX-BEARING ORGANISMS

Species	MLD (MU/20 g)
TTX-bearing organisms	
Xanthid crab *Atergatis floridus*	1000
Tropical goby *Yongeichthys criniger*	>300
Japanese newt *Cynops pyrrhogaster*	>10,000
Puffer fish	
Toxic	
Takifugu niphobles	700–500
Takifugu pardalis	500–550
Takifugu rubrips (culture)	300–500
Generally nontoxic and rarely toxic	
Lagocephalus wheeleri	15–19
Lagocephalus gloveri	19–20
Liosaccus cutaneus	13–15
Nontoxic	
Boxfish	0.9–1.3
Non-TTX-bearing organisms	
General fish	
Oplegnathus punclatus	0.8–0.9
Oplegnathus fasciutus	0.8–1.8
Girella punctata	0.3–0.5
Land animal	
Mouse	1

Source: Saito *et al.* (1984).

and pathogenic microorganisms in TTX-bearing animals, resulting in utilization as potent pain-stopper medicine for rheumatism, neuralgia, and heavy cancer, and is expected as a promising anesthetic.

TTX-bearing organisms such as puffers, gobies, xanthid crabs, newts, and gastropods have very high resistibility against TTX in comparison with that of non-TTX-bearing organisms such as fish, mice, and so on, as shown in Table XIX (Koyama *et al.*, 1983; Saito *et al.*, 1984). TTX-bearing organism should be qualified to have high TTX resistibility on TTX intoxication from a food chain. When puffers are cultured in a net during growing up to adult since fertilization, puffer grown are never intoxicated with TTX. However, such nontoxic puffers keep high TTX resistibility yet and can be intoxicated if TTX-containing foods are fed to them as shown in Table XX. On the other hand, in a case of non-TTX-bearing fish, which have not high TTX resistibility even though less than MLD_{50} amount of TTX-containing foods continues to

TABLE XX
TTX INFESTATION TO NONTOXIC CULTURED PUFFER FISH BY FEEDING ON TTX-CONTAINING LIVER OF WILD PUFFER FISH

Dosage of TTX	Toxicity score of liver (MU/g)	
Feeding periods (days)	0.5-MU/g body weight per day	4-MU/g body weight per day
20	<4	<4
40	<4	6
60	<4	90
80	<4	95
100	11	100
120	29	140
140	37	210
200	70	420
240	70	480

be fed to them for a long time, they never accumulated TTX in the liver and exclude or decompose it. These different phenomena between animals may come from each inherited quality.

The reason why they have high resistibility against TTX has been suggested to have a lot of special TTX-resistant (TTX-R) sodium channels, different from those of non-TTX-R organisms. As being suggested, two different types of sodium channels were surely found. One, TTX-R sodium channel is surely 3300 times higher TTX-R than that of another "TTX-sensitive sodium channels." However, since TTX-R sodium channels distribute not only puffers but also non-TTX-bearing organisms, its number in puffers seems to be not richer to deserve high TTX resistibility. Therefore, species-specific mechanism of masking and/or modulating TTX in TTX-bearing organisms seems to be equipped in the TTX-bearing animals. Probably functional group involved in toxicity in TTX structure may bind to some high molecular weight substance which is reversible. Herein, the above high molecular weight substance is suggested to occur only in TTX-bearing organisms. On stimulation, TTX is secreted alone and/or with TTX-binding substance and plays a role of defense and/or offense substance. Such a TTX-binding high molecular weight substance seems to function as follows: The shore crab, *Hemigrapsus sanguineus*, is highly resistant to TTX. To elucidate the mechanism of TTX resistance, its body fluid was examined for neutralizing effects against TTX (Shiomi *et al.*, 1992). When the body fluid was injected into mice together with TTX, the lethal activity of TTX was greatly reduced. However, the body fluid did not counteract the lethal effect of PSPs. The body fluid contains TTX-binding,

high molecular weight substances (>2,000,000) that are responsible for the neutralizing activity against TTX.

2. *Mechanism of action on sodium channels*

Although TTX blocking the nerve and muscle conduction had been known for many years (Kao, 1966; Narahashi, 1974), it was in 1960 that the sodium channel was focused as the possible action site of TTX. As known, TTX could block the action potential without any effect on the resting membrane potential and the delayed rectification because of the activity of potassium channels. Hence, TTX is suggested to block the sodium channel (Narahashi *et al.*, 1960). This hypothesis was extensively elucidated by the voltage clamp technique using lobster giant axons (Narahashi *et al.*, 1964). Figure 16 is an example of such experiment. Sodium currents are completely and reversibly blocked by TTX while potassium currents are unchanged. Nowadays, TTX has become a popular chemical tool in the laboratory and is used as a useful biochemical agent. A lot of studies have been conducted about TTX action, including the possible effects on sodium channel receptors and other ion channels and dynamical mechanism of sodium channel blocks. There have been published reviews describing the mechanism of action of TTX

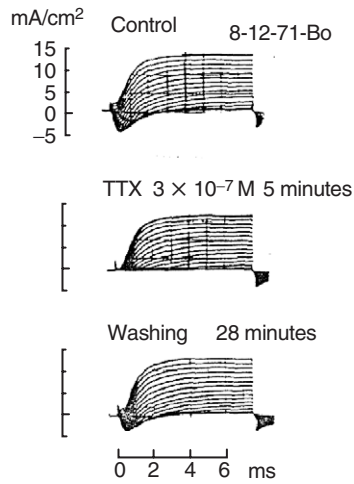


FIG. 16 Families of membrane currents associated with step depolarization (10-mV steps) in a squid giant axon before and during external applications of 3×10^{-7} M TTX and after washing with toxin-free medium. Note that TTX blocks transient sodium currents without any effect on steady-state potassium currents (Narahashi, 2001).

(Catterall, 1980, 1992, 1995, 2000; Narahashi, 1974, 1988a,b, 2001). Meanwhile, the structure and function of voltage-gated sodium channel have been clearly demonstrated in the past few years.

The sodium channel consists of various subunits, depending on the tissue of origin. The α subunit is the major and most important component because expression of the α subunits of brain or skeletal muscle sodium channels in *Xenopus* oocytes without the β subunits yields functional sodium channels, although these are activated and inactivated more slowly than the sodium channels in native neuron or muscle. Only when coexpressed with β_1 and β_2 subunits, normal channel gating is observed (Isom *et al.*, 1992, 1995; Schreibmayer *et al.*, 1994). The channel from mannalian brain is a complex of an α subunit (260 kDa), a β_1 subunit (36 kDa), and a β_2 subunit (33 kDa). The channel from skeletal muscle contains only the α and β_1 subunits; in the channel from electric eel electroplax, only the α subunit is required for ion conductance.

3. *Molecular structure of sodium channels*

The α subunit contains four homologous domains (*I–IV*) and each domain consists of six α-helical transmembrane segments (S1–S6) (Catterall, 1992, 1995; Guy and Seetharamulu, 1986; Narahashi, 2001). A loop that dips into the transmembrane region of the protein between transmembrane segments S5 and S6 of each domain is considered to form a channel pore. Within the pore, a set of four amino acids (aspartic acid, glycine, lysine, and alanine), occupying equivalent positions in each of the four domains, come together to form a narrow bottleneck and are likely intimately involved in channel conductance and ion selectivity (Denac *et al.*, 2000). The S4 segments carry multiple positive charges by a number of conserved arginine or lysine residues occupying every third position along the chain interspersed by two hydrophobic residues. These charges physically traverse the membrane in a corkscrew manner in response to the electric field, and are believed to form the voltage sensor. The negative internal transmembrane electric field pulls these charges into the membrane, locking them into a "cocked" position. Current models indicate that these positive charges be stabilized within the membrane segments. Depolarization releases the S4 segments to "unscrew" outward, initiating a conformational change that opens the channel (Catterall, 2000). A small cytoplasmic loop between domains *III* and *IV*, containing the hydrophobic amino acids isoleucine, phenylalanine, and methionine, comprises the fast inactivation gate. These amino acids act as a "tethered ball," which physically blocks the pore by binding to receptor residues in the cytoplasmic opening in response to conformational changes linked to channel activation.

The β subunits could indirectly modulate channel gating and ion conductance by stabilizing relevant α subunit conformations. They also possess immunoglobulin-like folds in their secondary structure analogous to cell-adhesion molecules. So they may interact with extracellular proteins through these folds and then influence the location and density of sodium channels in neural tissues (Bonhaus *et al.*, 1996; Catterall, 2000).

The sodium channel is the target for several classes of natural neurotoxins. These toxins may affect channel function in a variety of ways by binding to specific receptor sites on the α subunit and interacting with specific portions of the channel protein. These toxins have been invaluable probes for channel structure and function and their specific sites have, in some cases, been localized on the channel protein. The inactivation gate is located in the inner loop between S6 of domain *III* and S1 of domain *IV*; transmembrane segment S4 of each domain contains voltage sensors; and several phosphorylation sites by protein kinase and protein kinase C are identified in the inner loops between domains *I* and *II* and between domains *III* and *IV* (Figure 17).

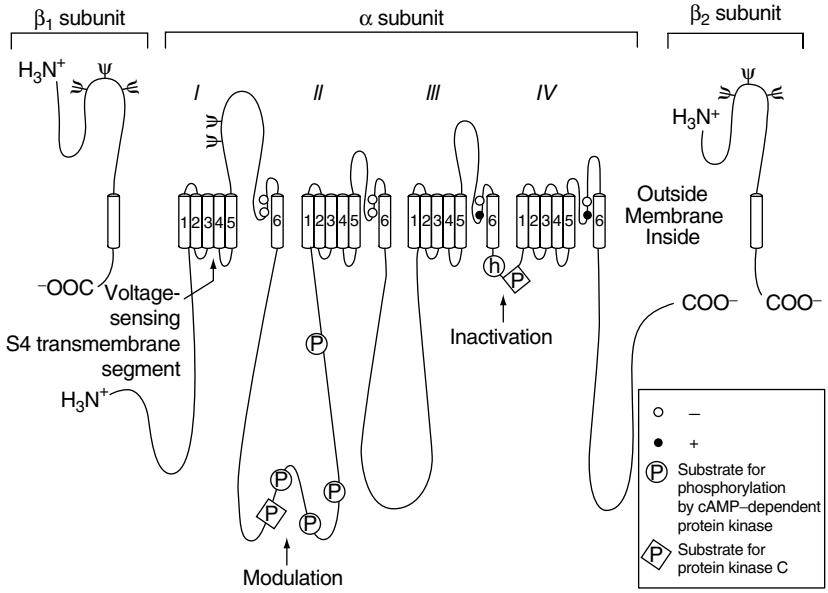


FIG. 17 Subunit of voltage-gated Na^+ channel α subunit consists of the four homologous domains (*I–IV*). The polypeptide chains are represented by continuous lines in each segment ion of the channel protein (Catterall, 2000; Narahashi, 2001).

4. *TTX-R sodium channels*

Most sodium channels in neurons and skeletal muscle fibers possess high sensitivity to TTX block. The IC_{50} values are in the order of nmol/liter. While those possessing less sensitivity or resistibility to TTX have also been found in various tissues. Denervated skeletal muscle and cardiac muscle represent classical cases. The IC_{50} values for TTX block are 1 μmol/liter for rabbit Purkinje fibers (Cohen *et al.*, 1981), 9 μmol/liter for rat myocardial cells (Matsuki and Hermsmeyer, 1983), and 14 μmol/liter for pig papillary muscle (Baer *et al.*, 1976). These IC_{50} values are approximately two to three orders of magnitude higher than those for TTX-sensitive (TTX-S) sodium channels of neutral tissues and skeletal muscle. Interestingly, TTX-R sodium channels are also found to be present in various neurons, including bullfrog sensory neurons (Campbell, 1988; Guo and Strichartz, 1990), garter snake sensory neurons (Jones, 1986), group C sensory neurons (Bossu and Feltz, 1984), rat nodose neurons (Ikeda and Schofield, 1987; Ikeda *et al.*, 1986), and human and mouse dorsal root ganglion (DRG) neurons (Kostyuk *et al.*, 1981; Matsuda *et al.*, 1978; McLean *et al.*, 1988; Schwartz *et al.*, 1990; Yoshida *et al.*, 1978).

In recent decade, TTX-R sodium channels were further focused because they are related to C fibers, which transmit pain sensation to the brain. If a chemical acts on TTX-R sodium channels but does not act on TTX-S sodium channels, this chemical is supposed to be a useful antinociceptive. The fact indicated that capsaicin blocked unmyelinated C fibers of human sural nerve *in vitro* and there was a good correlation between the sensitivity of C fibers to capsaicin and their resistance to TTX. The C fiber action potentials were found to be completely blocked by capsaicin in the presence of TTX (Grosskreutz *et al.*, 1996).

The characteristics of TTX-S and TTX-R sodium channels of rat DRG neurons are as follows (Narahashi, 2001; Ogata and Tatebayashi, 1992; Roy and Narahashi, 1992). The IC_{50} values for TTX block are 0.3 nmol/liter and 100 μmol/liter for TTX-S and TTX-R sodium channels, respectively. Thus, there is a 300,000-fold difference in TTX sensitivity between the two types of sodium channels. The kinetics of sodium current are markedly different, TTX-S sodium currents are faster than TTX-R sodium currents in both activation and inactivation phases. Significant differences are also found in the activation and inactivation voltages. The potentials for 50% activation of sodium current are –26 mV for TTX-S and –15 mV for TTX-R channels and those for 50% inactivation are –70 mV for TTX-S and –40 mV for TTX-R channels.

TTX-S and TTX-R sodium channels are known to have significant differences in sensitivity to certain chemicals. Both chemicals lead and cadmium

decreased the sodium currents and changed more activation in TTX-R than TTX-S sodium channels. The lead-induced changes of the potential for 50% conductance are 15 mV and 25 mV, and the lead-induced decreases in maximum conductance are 8% and 55% for TTX-S and TTX-R sodium channels, respectively. The cadmium-induced changes of the potential for 50% conductance are 25 mV and 21 mV, and the cadmium-induced decreases in maximum conductance are 16% and 69% for TTX-S and TTX-R sodium channels, respectively.

On the other hand, synthetic pyrethroid insecticides tetramethrin and allethin were found to significantly affect the sensitivity of TTX-S and TTX-R sodium channels (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994). Tetramethrin reveals an enormous tailing current responsible for terminating a depolarizing pulse in a concentration-dependent manner, while causing almost no effect on the peak sodium current. Pyrethroids modulate the individual sodium channel by prolonging the open time and by inhibiting the activation (Chinn and Narahashi, 1986; Yamamoto *et al.*, 1983), and a method has been developed to calculate the percentage of sodium channels modified by pyrethroids (Tatebayashi and Narahashi, 1994). Comparison of TTX-S and TTX-R sodium channels for their percentages of tetramethrin modification as a function of concentration is given in Table XXI (Narahashi, 2001). TTX-R sodium channels are 30–100 times more sensitive to tetramethrin than TTX-S sodium channels.

Sodium channels from different organs have been cloned and classified. Some structural aspects of sodium channels are being disclosed

TABLE XXI

PERCENTAGES OF THE FRACTION OF TTX-S AND TTX-R SODIUM CHANNELS MODIFIED BY VARIOUS CONCENTRATION OF TETRAMETHRIN

	Modification of sodium channels (%)	
Tetramethrin (μM)	TTX-S	TTX-R
0.01		1.31 ± 0.28
0.03	0	5.15 ± 0.30
0.1	0.24 ± 0.10	15.35 ± 0.79
0.3	1.25 ± 0.13	35.48 ± 2.70
1.0	3.53 ± 0.66	57.82 ± 2.29
3.0	7.70 ± 1.20	74.85 ± 1.23
10.0	12.03 ± 1.89	81.20 ± 1.57

Each value indicates the mean ± S.E.M. ($n = 4$).
Source: Narahashi (2001).

(Catterall, 1992, 1995; Goldin, 1999; Marban *et al.*, 1998; Plummer and Meisler, 1999). Sodium channels in the rat brain are classified into type I, II, IIA, and III, which comprise the α, β_1, and β_2 subunits, and all are sensitive to TTX block (IC_{50} values in the order of nmol/liter). In adult skeletal muscle, μl or SkM sodium channels are found to contain α and β subunits. They are sensitive to nmol concentrations of TTX and also sensitive to the blocking action of μ-conotoxin GIIIA. In the heart and denervated skeletal muscle, HI or SkM2 sodium channels are found to be resistant to TTX with IC_{50} values in the order of 2–6 μmol/liter. In neuroendocrine and peripheral neurons, PN1 sodium channels are also sensitive to TTX with IC_{50} values in the order of nmol/liter. In dorsal root and trigeminal ganglion neurons, SNS or PN3 and SNS2 or PN5 sodium channels are found. Among them, SNS sodium channels are insensitive to TTX with IC_{50} values in the order of 30 μmol/liter or more, whereas SNS2 sodium channels are resistant to TTX with IC_{50} values in the order of 1 μmol/liter.

5. *Site of action and binding of TTX*

Chemical compounds that block the sodium channels can be classified into several groups based on binding sites (Catterall, 1992). TTX, STX, and μ-conotoxin bind to site 1 of sodium channels causing channel block. Batrachotoxin, grayanotoxins, veratridine, and aconitine, which prolong the sodium channel opening, bind to site 2. Site 3 is bound by sea anemone and α-scorpion toxins (class 1), which also cause a prolongation of sodium channel opening. β-Scorpion toxins of classes 2 and 3 bind to site 4, resulting in modulation of channel gating. Brevetoxins and ciguatoxin bind to site 5, prolonging sodium channel opening. Pyrethroids which also prolong sodium channel opening and modulate the gating bind to site 6.

Binding site 1 of sodium channels for TTX locates in short segments SS1 and SS2 that connect transmembrane segments S5 and S6 of each domain (Catterall, 2000). This part plays as the selectivity filter for various ions, and TTX blocks the selectivity filter in the outer pore of sodium channels (Hille, 1975). Amino acids with negative charge are present in analogous positions in all four domains. These amino acids are supposed to form outer and inner rings that serve as the binding site for TTX (Catterall, 2000). Cardiac sodium channels are 200–1000 times less sensitive to TTX than TTX-S neuronal sodium channels. This difference is caused by various amino acids. The brain and skeletal muscle sodium channels have phenylalanine and tyrosine, respectively, in the P-loop of domain *I*, whereas the cardiac sodium channels contain cysteine in the corresponding position (Backx *et al.*, 1992; Heinemann *et al.*, 1992; Penzotti *et al.*, 1998; Satin *et al.*, 1992; Sunami *et al.*, 2000). The sodium channels of DRG (PN3/SNS) are more resistant to TTX than

the cardiac sodium channels (Roy and Narahashi, 1992). These channels contain serine in domain *I* to substitute phenylalanine in cardiac sodium channels (Sivilotti *et al.*, 1997).

G. THERAPEUTIC APPLICATION OF TTX

Since TTX was found to effectively block sodium channel, it has been conducted for the possible therapeutic applications. One possible application is a neuroprotective drug in the treatment of ischemic damage of the brain that follows stroke. Whereas the onset of stroke is accompanied by a rapid local damage in the brain, infarction spreads from the point of damage slowly. Thus, there is a therapeutic window during which damages to the peri-infarct area can be prevented or minimized by administration of a neuroprotective drug (Koroshetz and Moskowitz, 1996).

Ischemia causes membrane depolarization, which in turn evokes repetitive discharges. At the nerve terminals, these discharges open calcium channels, causing influx of Ca^{2+} which in turn releases neurotransmitter. At the glutamatergic synapses, the released glutamate activates the NMDA receptor channel through which Ca^{2+} ions enter. The increase in intracellular Ca^{2+} concentration kills the neuron. Therefore, the presynaptic sodium channels, the presynaptic calcium channels, and/or the NMDA receptors may be blocked to prevent or minimize the ischemic damages.

TTX is effective in mitigating ischemic damages caused by occlusion of vessels in rat hippocampus (Yamasaki *et al.*, 1991) and those caused by exposure to veratridine in cerebellar neurons and hippocampal neurons (Lysko *et al.*, 1994). Focal microinjection of TTX reduces neurological deficits and tissue loss after spinal cord injury. In rats subjected to a standardized weight-drop contusion, the injection of TTX into the injury site was effective in attenuating the damages to the large diameter axons (Rosenberg *et al.*, 1999).

TTX is also effective in blocking electrographic seizures *in vitro* (Burack *et al.*, 1995). Stimulus train-evoked seizures were blocked for several hours after localized injection of 50 μmol/liter TTX in rat hippocampal slices, whereas responses to single stimulus were minimally altered by TTX. TTX injections that blocked electrographic seizures were nearly always located in CA2/3 stratum radiatum and/or stratum lacunosum-moleculare. When applied in the perfusion medium, TTX was effective in blocking electrographic seizures at low concentrations (5–20 nmol/liter). TTX was also effective in preventing posttraumatic epileptogenesis in rats (Graber and Prince, 1999). Evoked epileptiform field potentials were observed in the injured cortex, and thin sheets of Elvax polymer containing TTX implanted over lesions were effective in decreasing evoked epileptiform potentials.

To find out drugs that could recover the intoxication caused by TTX, some attempts were made. 4-Aminopyridine at 1–2 mg/kg (i.m.) was found to be effective in guinea pigs to restore the toxin-induced diaphragmatic block, bradypnea, bradycardia, and depressed cortical activity (Chang *et al*., 1996, 1997).

TTX was a good anesthetic to the rabbit eyes without causing any systemic toxicity when operating an excimer laser keratectomy (Chang *et al*., 1997). Topically applied TTX at 1 or 10 mmol/liter was shown to be a long-acting anesthetic in the rabbit cornea (Schwartz *et al*., 1998a,b). Subcutaneous injections of TTX with epinephrine were effective for prolonged local anesthesia of poreutaneous sciatic nerve in rats. The median effective concentration of TTX was 11.5 μmol/liter and reversible blocks lasted over 13 hours (Kohane *et al*., 1998).

Recently, TTX is found to have a renoprotective action in a rodent model for ischemia reperfusion in jury that contributes significantly to posttransplant graft dysfunction (Garvin *et al*., 1999). TTX was also useful to protect the kidney from warm ischemia in uninephrectomized rats (Li *et al*., 1992). A Mab against TTX has been developed. Mice were injected i.p. with 1.5 MU of TTX, and 3 minutes later 100 μg of antibody immunoglobulin G (IgG) were injected through the tail vein. A 100% survival was observed (Matsumura, 1995a). The Mab was highly specific for TTX, and no cross-reaction to TTX derivatives and paralytic shellfish toxins, and could neutralize TTX *in vitro* (Matsumura, 1995b). The ability of TTX-specific Mab to confer passive protection against lethal TTX challenge was investigated (Rivera *et al*., 1995). The Mab, T20G10, was specific for TTX, less reactive with anhydroTTX, and unreactive with TDA. T20G10 specifically inhibited TTX binding, but had no effect on STX binding. T20G10 prevented death of mice orally administered with TTX.

IV. HIGHLIGHT OF VIEWPOINT

A. PROPOSED PROGRAMS OF NEW FOOD INDUSTRY FOR PUFFER

1. Production of nontoxic puffer by new developed biotechnology

The issue "how to make wild puffer safe to eat" has been carefully elucidated in the section on prevention. Now, harvest of wild puffer in Japan has recently outstandingly decreased on account of their overfishing since 10 years ago. Instead, puffer culture has increased resulting in 80% of total marketed puffer.

For their culture, the puffer parents are paired with toxic wild female and nontoxic cultured male puffer. The eggs fertilized with nontoxic sperms are toxic

in the beginning and become nontoxic larvae after 2 weeks of hatching. These larvae grow into adult by feeding nontoxin diet in surrounding nets. These adult puffers are nontoxic. Puffer fan can eat each part of them with safety.

2. *Revival of a traditional food puffer liver "Kimo"*

Since Japanese Public Health Administration prohibited serving puffer liver, "kimo" as food in 1983, "kimo" (a traditional food) fans have been anxious to revive safe "kimo." The kimo harvested in the above culture will be available for revival of a traditional food ("kimo"), which is expected to activate Japanese fishery industry because puffer livers have been discarded so far (Noguchi *et al.*, 2004).

B. METHOD OF SPECIES IDENTITY FOR PUFFER BY GENOME TECHNIQUES

1. *Introduction*

Species identification of seafood product is important for the implementation of the labeling regulations as set by many countries. These regulations to prevent the substitution of some commercially important fish can be effectively achieved when species-specific data of all fish species are available. Genome and protein techniques are the two valuable methodologies that can be used for fish species identification. These methods can prevent adulteration of toxic puffer species as nontoxic puffer one. Hence, the development of genome and protein techniques for effective identification of puffer species is critically needed.

Recently, molecular methods based on nucleic acids amplification by means of polymerase chain reaction (PCR) have been well established and applied to the field of species identification. Usually, PCR is coupled with other techniques that are capable of detecting differences in the sequences of the PCR amplification products. With the PCR products, restriction fragment length polymorphism (RFLP), or single-strand conformation polymorphism (SSCP) could achieve analyses. Species identification can also be accomplished by using the other means of PCR reaction, such as random amplified polymorphic DNA (RAPD). All these techniques use gel-based patterns to facilitate species identification. They could be divided into two categories based on the targets: the RAPD is directed at multiple gene locations, while the RFLP and SSCP are generally aimed at only one or very few gene locations (Hold *et al.*, 2001). These methods for species identification are chiefly based on mitochondrial cytochrome *b* gene (Quinteiro *et al.*, 1998; Russell *et al.*, 2000), mitochondrial 12S rRNA (Comesana *et al.*, 2003), mitochondrial 16S rDNA

(Karaiskou *et al.*, 2003a), mitochondrial ND-1 gene (Politov *et al.*, 2000), mitochondrial α-actin gene (Fernandez *et al.*, 2000), and nuclear 5S rDNA (Carrera *et al.*, 2000; Karaiskou *et al.*, 2003a,b). Mitochondrial DNA is the primarily employed genetic tool, and one of its advantages is the high copy numbers of the mitochondrial genome (Mackie *et al.*, 1999), compared to nuclear genome within a cell. The great advantage of the DNA techniques is that there is satisfactory information to be recognized by the primers although the DNA degrades on processing. The principal theory and practical application of these methods for species identification are concisely discussed below.

2. *Direct sequence analysis*

Direct sequencing of species-specific DNA is currently the most reliable approach for species identification. However, it was regarded as a labor-intensive and high-priced technique (Chapela *et al.*, 2003). The most important advantage of this technique is that the results are not affected by intraspecific variability (Bossier, 1999). Moreover, it is not necessary to investigate the data from all reference samples because the sequence of nucleotide residues can be compared to those in the international database GenBank. This technique has been successfully applied in identifying many fish species, including cephalopod (Chapela *et al.*, 2003), caviar (Ludwig *et al.*, 2002), tuna (Lockley and Bardsley, 2000; Takeyama *et al.*, 2001), *Trachurus* species (Karaiskou *et al.*, 2003a,b), sole and halibut (Cespedes *et al.*, 2000), puffer (Cheng *et al.*, 2001; Hsieh, 2003; Hwang *et al.*, 2002a; Song *et al.*, 2001), flatfish (Comesana *et al.*, 2003), anchovy and sardine (Jerome *et al.*, 2003; Sebastio *et al.*, 2001), hake (Quinteiro *et al.*, 2001), and salmon (Russell *et al.*, 2000). The complete DNA sequence also provides an invaluable tool for accomplishing genetic studies among taxa and phylogenetic relationships for closely related fish species (Hsieh, 2003; Karaiskou *et al.*, 2003a,b). When this technique was applied to identify the tuna and bonito species of canned products using the amplified product of a 59-bp short fragment, 9 of the 11 samples were correctly differentiated (Unseld *et al.*, 1995). The increased use of nucleotide sequences in PCR and direct sequencing for elucidating species-specific genes or full genome information in vertebrates has made international databases available, complementary for subsequent comparative studies in species identification of fish.

3. *PCR-RFLP*

At present, PCR-RFLP is the most common DNA-based technique used for fish species identification. Scientists draw the greatest attention to mitochondrial cytochrome *b* gene. The species-specific gene appears to have substantial inter- and intraspecies variation in its original nucleotide sequence, and the

level of variation within species is much less than between species (Mackie *et al.*, 1999). The technique is considered as an easy, rapid, and relatively cheap tool in identifying fish species. PCR-RFLP allows for the amplification of a conserved region of DNA sequence using PCR and the detection of the specific patterns after treatment with restriction enzymes, usually 4–6 bp in length (Hold *et al.*, 2001; Sun and Lin, 2003). Generally, the shorter recognition sequence of enzyme will generate greater number of fragments (Sotelo *et al.*, 2001). Direct sequencing technique is generally considered to be the preliminary step for RFLP analysis. The generated sequences are then utilized for examination of potential restriction sites of nucleases that could be applied for the differentiation of each species. Finally, the species-specific patterns of samples obtained after the cleavage by restriction enzymes could be detected and compared after electrophoresis (Ludwig *et al.*, 2002). In recent years, many reports showed the great effectiveness of PCR-RFLP analysis in differentiating raw fish species (Cespedes *et al.*, 2000; Comesana *et al.*, 2003; Fernandez *et al.*, 2000; Karaiskou *et al.*, 2003a,b; Politov *et al.*, 2000; Russell *et al.*, 2000; Sebastio *et al.*, 2001; Takeyama *et al.*, 2001) and cooked fish or seafood products (Chapela *et al.*, 2003; Hold *et al.*, 2001; Jerome *et al.*, 2003; Ludwig *et al.*, 2002; Mackie *et al.*, 1999; Quinteiro *et al.*, 2001). Except for the 12S rRNA (Cespedes *et al.*, 2000; Comesana *et al.*, 2003), 16S RNA (Colombo *et al.*, 2002), α-actin (Fernandez *et al.*, 2000), control region, and ND-1 gene (Politov *et al.*, 2000; Quinteiro *et al.*, 2001), most of these publications concentrate on the mitochondrial cytochrome *b* gene. As to the severely degraded products like sterilized food, the mitochondrial control region is the most sufficient for the identification of fish species (Mackie *et al.*, 1999; Quinteiro *et al.*, 2001). PCR-RFLP was shown to achieve the recognition of mixed species of canned tuna fish, while PCR-SSCP was not (Mackie *et al.*, 1999). With this technique, a 96% success rate was achieved for authentic identification from a total of 120 examinations (Hold *et al.*, 2001). Typically, the use of two or more restriction enzymes was adequate to accurately discriminate fish species. In some cases, PCR-RFLP did not allow precise identification of all the test fish species (Colombo *et al.*, 2002; Sotelo *et al.*, 2001). So far, intraspecific variation has not been a serious problem when using this technique for fish species identification, although this possibility cannot be excluded.

4. PCR-SSCP

SSCP is a fast, easy to perform, and well-suited technique for detecting even single base changes in short DNA fragments (Hayashi, 1996). PCR is initially applied to amplify the region of interest. The resultant DNA is usually heated in a denaturing solvent and separated as single-strand DNA

by electrophoresis in a nondenaturing polyacrylamide gel electrophoresis (PAGE) (Orita *et al.*, 1989). In comparison with PCR-RFLP and RAPD-PCR, PCR-SSCP is the best method to reflect the entire nucleotide sequence of the sample of interest (Orita *et al.*, 1989). From a total of 72 cases in identifying canned tuna fish, only 7 cases were incorrectly identified (Rehbein *et al.*, 1999). The PCR-SSCP of mitochondrial 12S rRNA allows for the identification of grouper species, wreck fish, and Nile perch fish (Asensio *et al.*, 2001). The weakness of the technique is that both the references and test samples need to be run on the same gel. In addition, limited information on SSCP patterns is available (Rehbein *et al.*, 1997).

5. *RAPD-PCR*

PCR is frequently used to amplify a particular DNA of interest. However, the RAPD analysis, also known as arbitrary primed PCR (AP-PCR), uses short primers amplifying fragments of DNA which are essentially unknown to the scientists (Dinesh *et al.*, 1996). It is a relatively faster, cheaper, and simpler technique for use than PCR-SSCP and PCR-RFLP (Mackie *et al.*, 1999). It can be done without having adequate information about the genome of the studied species. Reproducibility is an encountering problem, but the use of strict PCR conditions and high quality of template DNA helps prevent the main drawback of this technique. Four of 20 10-mer random oligonucleotide primers were applied to identify part of mussel species using RAPD method. Further use of the designed primers based on the information could lead to achieving complete identification of the mussel species (Rego *et al.*, 2002). Callejas and Ochando (2001) used 10 random primers to create RAPD markers for identification of barbell fish. However, this approach failed to identify canned tunas and bonitos (Mackie *et al.*, 1999). The use of RAPD in 16S rRNA gene failed to identify all the test puffer fish species because a low-level variation in DNA sequence was observed between *T. rubripes* and *T. pseudommus* (Song *et al.*, 2001).

6. *Puffer genome*

The international Human Genome Project that aims to identify and characterize all human genes in order to facilitate the knowledge of human biology has provided a driving force to vertebrate genomics research (Lander *et al.*, 2001; Venter *et al.*, 2001). Identifying coding and regulatory sequence is a major challenge of the postsequencing era of the human genome project. Comparative genomics, the comparison of genomic sequences from different species, is a good approach to interpret the human genome. Therefore, several other vertebrate genomes are being investigated to gain insight into their biology and to serve as models. These models include mouse, rat,

chicken, frogs, and fish such as the zebrafish, medaka, and fugu (Amaya *et al.*, 1998; Beier, 1998; Venkatesh *et al.*, 2000; Waterston *et al.*, 2002). Among them, the "torafugu," *T. rubripes*, has the smallest vertebrate genome of about seven to eight times smaller than human's but has a similar repertoire of genes. Its genes are densely packed with short intergenic and intronic sequences and contain less than 10% repetitive DNA (Brenner *et al.*, 1993). Its usefulness in the discovery of conserved regulatory elements has already been elucidated. Hence, the puffer genome is a good complement to the genetic studies in other vertebrates (Elgar *et al.*, 1996; Venkatesh *et al.*, 2000). The whole genome of *T. rubripes* was identified as 365 Mb (Aparicio *et al.*, 2002). The authors also highlighted that three-quarters of the predicted human proteins have strong match to fugu proteins, implying that there exists an interesting linkage between human and fugu proteomes (Aparicio *et al.*, 2002). For the most updated puffer fish genome sequences, some Web sites such as http://fugu.hgmp.mrc.ac.uk, http://www.fugu-sg.org, and http://www.jgi.doe.gov/fugu are available.

C. METHOD OF SPECIES IDENTITY FOR PUFFER BY PROTEIN TECHNIQUES

Due to morphological similarities, the manufacturers and consumers may have difficulty in distinguishing nontoxic *L. gloveri* from *L. lunaris*, a species that accumulates lethal level of TTX in its muscle. Therefore, serious food poisoning incidents due to ingestion of toxic puffer fish or the toxic dried dressed fish fillets have occasionally occurred in Taiwan. From the viewpoint of food protection and public safety, it is critical to accurately identify the nontoxic puffer fish from the toxic species. Sodium dodecyl sulfate-PAGE (SDS-PAGE), native isoelectric focusing (N-IEF), and immobilized pH gradients-two-dimensional electrophoresis (IPG-2DE) were employed to validate the feasibility of using these techniques to develop species-specific muscle protein profiles for puffer species identification. Species-specific bands of sarcoplasmic, myofibrillar, SDS-soluble, and urea-soluble proteins were found in the molecular weight region below 30 kD of the SDS-PAGE patterns. Coomassie blue/silver double staining provided better protein banding patterns for discrimination of different puffer species than Coomassie blue staining alone. In some cases, especially for Coomassie blue-stained sarcoplasmic proteins, the density of the protein profiles facilitates precise identification. The use of double staining to stain SDS-soluble proteins seemed to be the best combined approach for identifying puffer species when using the SDS-PAGE method (Chen and Hwang, 2002; Chen *et al.*, 2002a,b). The SDS-PAGE pattern of 1% SDS extract with double staining and the concentration of low-molecular-weight proteins are shown in Table XXII. In the region above 19.5 kD, the pattern was almost the same

TABLE XXII

COMPOSITION PERCENTAGE OF 1% SDS EXTRACT WITH LOWER MOLECULAR WEIGHT (≤30.5 kD) FROM SEVEN PUFFER SPECIES FOLLOWING DOUBLE STAINING AND DETERMINED BY DENSITOMETRY

Molecular weight (kD)	Relative amount (%)						
	LW	LG	LL	LI	TO	TX	SP
30.5	4.9 ± 0.5^{a}	3.9 ± 0.8^{a}	3.9 ± 1.0^{a}	4.0 ± 0.6^{a}	–	–	–
30.2	–	–	–	–	3.6 ± 1.0^{a}	4.0 ± 0.6^{a}	1.3 ± 0.3^{b}
29.3	–	–	–	–	2.3 ± 0.6^{a}	3.7 ± 1.0^{a}	–
28.4	–	3.5 ± 0.8	–	–	–	–	–
28.0	3.6 ± 1.2^{a}	–	3.8 ± 0.6^{a}	3.7 ± 1.0^{a}	1.5 ± 0.2^{b}	0.4 ± 0.2^{c}	3.6 ± 1.0^{a}
26.8	–	–	–	–	3.8 ± 1.0^{a}	2.8 ± 0.6^{a}	1.1 ± 0.2^{b}
24.9	4.0 ± 1.2^{ab}	5.4 ± 0.8^{a}	–	3.8 ± 0.6^{b}	–	–	–
24.2	–	–	3.7 ± 0.8	–	–	–	–
23.9	–	–	–	–	4.1 ± 0.6^{a}	3.4 ± 0.7^{a}	4.1 ± 0.9^{a}
22.8	2.6 ± 0.4^{b}	3.8 ± 1.0^{a}	2.5 ± 0.9^{ab}	4.2 ± 1.0^{a}	–	–	2.0 ± 0.5^{b}
21.9	4.0 ± 1.4^{b}	1.8 ± 0.6^{c}	5.5 ± 0.8^{b}	5.7 ± 0.8^{b}	5.0 ± 1.3^{b}	6.2 ± 0.8^{b}	8.2 ± 0.7^{a}
19.5	3.3 ± 1.0^{ab}	2.9 ± 0.4^{b}	2.5 ± 0.4^{b}	4.2 ± 0.3^{a}	–	2.3 ± 0.8^{b}	–
18.9	–	–	–	–	3.6 ± 0.6	–	–
17.0	2.2 ± 0.3^{a}	–	1.9 ± 0.3^{a}	1.0 ± 0.2^{b}	–	–	–

16.6	–	–	–	–	–	–	1.6 ± 0.3
15.0	–	–	–	–	–	–	0.8 ± 0.2
13.9	4.2 ± 0.3[b]	6.5 ± 0.5[a]	2.4 ± 0.5[c]	2.5 ± 0.2[c]	2.4 ± 0.5[c]	1.6 ± 0.3[d]	–
11.5	–	–	–	1.5 ± 0.4	–	–	–
10.3	3.1 ± 0.5[a]	1.5 ± 0.3[b]	2.8 ± 0.7[a]	–	3.8 ± 0.5[a]	3.1 ± 0.5[a]	0.7 ± 0.2[c]
8.7	–	–	–	–	2.8 ± 0.5[a]	1.3 ± 0.3[b]	2.0 ± 0.4[a]
7.7	–	2.8 ± 0.5	–	–	–	–	–
7.4	3.5 ± 0.4[a]	–	–	2.3 ± 0.6[b]	–	–	–
7.1	–	–	3.7 ± 0.7	–	–	–	–

LW, *Lagocephalus wheeleri*; LG, *Lagocephalus gloveri*; LL, *Lagocephalus lunaris*; LI, *Lagocephalus inermis*; TO, *Takifugu oblongus*; TX, *Takifugu xanthopterus*; SP, *Sphoeroides pachygaster*.

Data are mean ± S.D. of triplicate assays, and "–" represents the relative amount being less than 0.1%. Valves in a rank not followed by a same letter are significantly different.

Source: Chen and Hwang (2002).

as that of Coomassie blue staining. In the double-stained region of 17.0–7.4 kD, the characteristic protein bands for each puffer were as follows: 17.0, 13.9, 10.3, and 7.4 kD for *L. wheeleri*; 13.9, 10.3, and 7.7 kD for *L. gloveri*; 17.0, 13.9, and 10.3 kD for *L. lunaris*; 17.0, 13.9, 11.5, and 7.4 kD for *L. inermis*; 13.9, 10.3, and 8.7 kD for *T. oblongus* and *T. xanthopterus*; and 16.6, 15.0, 10.3, and 8.7 kD for *Sphoeroides pachygaster*. *T. oblongus* and *T. xanthopterus* could be differentiated by the presence of 18.9 kD and 19.5 kD protein bands, respectively.

Evaluation of the native IEF patterns showed that the majority of water-soluble puffer muscle proteins fell in the region with isoelectric point (p*I*) values of 5.85–8.65. The characteristic species-specific protein bands were found in all the three regions of p*I* 3.50–5.20, 5.85–6.55, and 7.35–8.15. Among them, the p*I* 3.50–5.20 was the most suitable region for identifying species-specific proteins. Coomassie blue staining was shown to be more adequate than silver-staining method for revealing the protein profiles for the identification of puffer fish species. Table XXIII listed the characteristic bands in the three clustered regions for each of the six puffer species. The species-specific bands at the acidic region of p*I* 3.50–5.20 were p*I* 4.18 and 4.90 for *L. wheeleri*; 5.02 for *L. gloveri*; 5.09 for *L. lunaris*; 4.20 and 4.50 for *L. inermis*; 4.13 for *T. oblongus*; and 4.59 for *L. sceleratus.* At the region of p*I* 7.35–8.15, the species-specific protein bands were p*I* 7.47, 7.63, and 7.80 for *L. wheeleri*; 7.38, 7.47, 7.63, and 7.82 for *L. gloveri*; 7.69, 7.86, and 7.97 for *L. lunaris*; 7.50, 7.77, and 7.99 for *L. inermis*; 7.77 and 7.99 for *T. oblongus*; and 7.44, 7.51, and 7.68 for *L. sceleratus.* Many of the numerous protein bands in the third region of p*I* 5.85–6.55 also provided good differential characteristics for species identification. Therefore, the Coomassie blue-stained native IEF gels, which contain many sharp and species-specific protein bands, can be used as a valuable tool for puffer species identificaiton. In comparison between Coomassie blue staining and silver staining, silver-staining IEF gels produced high levels of background staining. Hence, native IEF is a feasible tool for identifying puffer species with either Coomassie blue stain or silver stain (Chen *et al.*, 2003).

In viewing the IPG-2DE patterns, it was noted that puffer muscle proteins that fell in the region with p*I* values of 3.5–7.0 and molecular weights of 7.4–45.0 kD were good for species comparison. The more acidic proteins of lower molecular weights showed species-specific characteristics. Therefore, species identification of puffer can be achieved from the comparison of IPG-2DE protein pattern profiles (Chen *et al.*, 2004).

Aside from conventional electrophoretic methods, capillary electrophoresis (CE) is considered as a novel electrophoretic technique for protein separation with advantages of being easy, rapid, automatic, using trace amount of test samples, and qualitative as well as quantitative within one analysis

TABLE XXIII

PROTEIN CONTENTS (mg/ml) AND THE SPECIES-SPECIFIC BANDS OF WATER-SOLUBLE PUFFER FISH PROTEINS WITH COOMASSIE BLUE STAINING

Fish species	Protein content	p*I* 3.50–5.20	p*I* 5.85–6.55	p*I* 7.35–8.15
Lagocephalus wheeleri	7.2	4.18, 4.90	5.90, 6.01, 6.08, 6.17, 6.28, 6.40, 6.43, 6.47	7.47, 7.63, 7.80
Lagocephalus gloveri	6.2	5.02	6.06, 6.12, 6.19, 6.41, 6.49	7.38, 7.47, 7.63, 7.82
Lagocephalus lunaris	6.3	5.09	5.90, 6.01, 6.45, 6.49, 6.54	7.69, 7.86, 7.97
Lagocephalus inermis	7.4	4.20, 4.50	5.89, 6.01, 6.45, 6.49	7.50, 7.77, 7.99
Takifugu oblongus	5.9	4.13	5.92, 6.01, 6.09, 6.33, 6.46, 6.53	7.77, 7.99
Lagocephalus sceleratus	10.4	4.59	6.14, 6.29, 6.41, 6.48, 6.55	7.44, 7.51, 7.68

Source: Chen *et al*. (2003).

(Grossman *et al.*, 1989). CE is a powerful tool for the separation of proteins and peptides since it could identify diverse protein products by only one amino acid residue. The main constitutes of a CE instrument are high-voltage power supply, eluent system, capillary column, detector, and data processor (Gordon *et al.*, 1988). It not only has the reliable separation theory from conventional electrophoresis but also the excellent separation resolution from chromatography (Kuhr, 1998). When applied for species identification, the pH of the eluent solvents (LeBlanc *et al.*, 1994), the protein amounts injected to the system (Gallardo *et al.*, 1995), and the packing material of the capillary column (Crockford and Johnston, 1995) all play important role in achieving success. CE also allows for precise identification of fish species that have been frozen, stored at different temperatures for several months (Larrain *et al.*, 2002; LeBlanc *et al.*, 1994). When compared to SDS-PAGE, the CE and 2DE had more discriminatory power for identifying the species of myosin light chains in tilapia (Crockford and Johnston, 1995). The CE profiles of the peptides and amino acids were effectively used to detect adulteration of shark fin (Chou *et al.*, 1998). SDS-capillary gel electrophoresis (SDS-CGE) has been established as a powerful qualitative and quantitative technique in separating fish myofibrillar proteins, and thus is considered as an alternative to classic SDS-PAGE for fish species identification (Sotelo *et al.*, 2000). The application of CE for puffer identification is worthy of study.

The focus of basic molecular research will gradually move from genes/genomes to proteins/proteomes because genome sequencing cannot define all protein components and their functions (Vihinen, 2001). To actually respond to the genes, the proteome referred to the systematic identification of the total protein complement of the genome must be done. Approaches for proteome analysis primarily include three protocols: 2DE, protein microchemistry, and bioinformatics. First of all, the IPG-2DE, which is the most commonly used procedure currently, is used to find out target proteins. Then matrix-assisted laser desorption time-of-flight mass spectrometry (MALD-TOF-MS) is used to characterize protein spots. Sequence tagging with tandem MS (MS–MS) can be employed to further identify the proteins. Finally, the established proteome results were compared with genomic and proteomic information from databases on the web (Babnigg and Giometti, 2004). So far, the application of proteomic protocols for the investigation of fish has been limited. Inspection of international databases for DNA and protein information shows that the relative amount of protein sequence information in aquatic vertebrate research is merely 4% of that of total DNA sequences (Pineiro *et al.*, 2003). Although the whole sequences of puffer genome has been established (Aparicio *et al.*, 2002), publications related to their proteomic information are still not available.

D. TTX AS ATTRACTANT AND HIBERNATION AGENT

We recently investigated the attracting effect of small dose of TTX on eight toxic small snail species (*P. didyma, N. lineata, N. vitellus, Z. sufflatus, N. clathrata, O. miniacea, O. mustelina*, and *O. hirasei*) and two nontoxic snail species (*Pomacea canaliculata* and *Satsuma bairdi*). It was found that all toxic snails showed significantly positive relationship between comparative attracting variation and the toxicity reported, and the relationship was a linear line (Hwang *et al.*, 2004). The relationship between TTX resistance ability and the toxicity also has a positive correlation. However, the nontoxic species showed the negative response. The more toxic snail appeared to be the more preferable to TTX, indicating that TTX is an attractant for toxic snails (Figure 18). Previous papers (Hwang *et al.*, 1990b,c, 1992b) have pointed that toxic gastropod possessed high-resistant ability to TTX and secreted the toxin as defense or attack agent. Matsumura (1995c) found that TTX was mostly distributed in the surface of puffer eggs, and might act as a pheromone to attract the male puffer. Meanwhile, Arakawa *et al.* (2003) investigated microdistribution patterns of TTX in the tissues of three species of puffer *T. vermicularis, C. patoca*, and *T. steindachneri*, and a Japanese

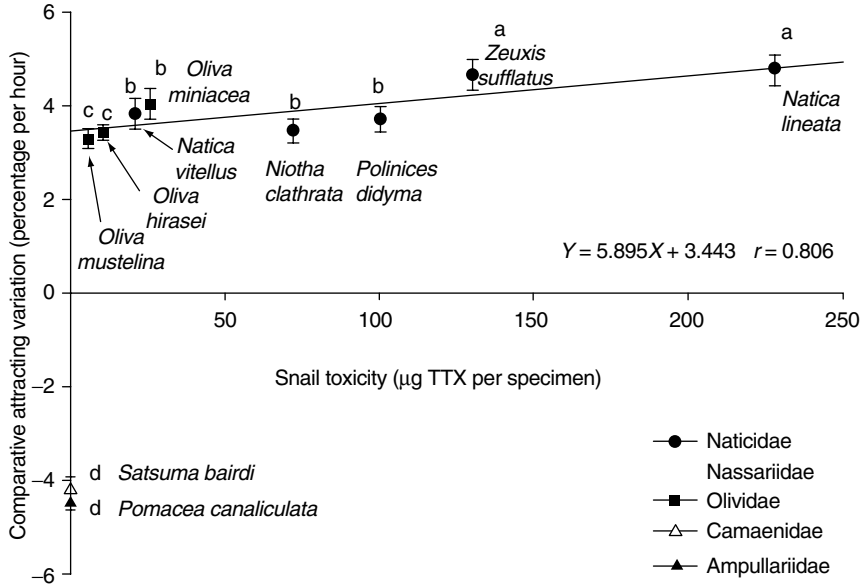


FIG. 18 The comparative attracting variation of TTX to toxic and nontoxic snails (Hwang *et al.*, 2004).

newt *C. pyrrhogaster* by means of a Mab-based immunoenzymatic technique under a light microscope. The distribution patterns in puffers varied in respect of species. In the skin sections of *T. vermicularis*, TTX was visualized at different shapes of glands in the epidermis layer, while in case of *C. patoca* and *T. steindachneri*, neither such gland nor gland-like apparatus was apparent, and TTX was mainly detected in sacciform cells. Similarly, in the ovary section of *T. vermicularis*, TTX was visualized at nucleus, yolk vesicles, and/or yolk granules in various stages (late pari nucleolus stage, yolk granule stage-I, and yolk granule stage-II) of oocytes. In *C. patoca*, TTX was localized at the connective tissues and in the nucleus of some perinucleolar oocytes. On the other hand, observation of the newt skin sections demonstrated that TTX was distributed at immature glands in juvenile and at the granular cells composing of granular and mixed glands in adult specimens. No specific stain of TTX was recognized in larval section. A duct-like structure extending from the gland toward super epithelial layer was visualized in the skin sections of both *T. vermicularis* and *C. pyrrhogaster*. When stimuli by wiping with gauze (''handling stimulus'') were given, these animals secreted an applicable amount of TTX from the skin, suggesting that they have a gland of TTX to secrete it toward the body surface possibly as a biological defensive agent. Hence, the biological significance of TTX in toxic animals should be further studied.

It is mysterious that TTX is assumed to act as major active agent of zombe powder used to kill people and then revive them. Haitian wizards usually use toxic puffer as a material of zombe powder, while the phenomenon of zombe is still a riddle or witchery. It is necessary to elucidate how much of toxin amount can let a person into hibernation and how to control time can let a person revive. If these problems become clear, TTX would be an important drug like anesthetic for healing serious burn wound, conducting organ transplantation, and intending space flight travel in future. On the other hand, circadian rhythms are driven by a pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus in mammals. Some reports have indicated that TTX reversibly abolishes the expression of circadian rhythm (Welsh *et al*., 1995; Yamaguchi *et al.*, 2003). Noguchi and Watanabe (2005) further showed that TTX resets the clock. Hence, the neurophysiological role of TTX in human needs further study.

V. SUMMARY

This chapter on TTX poisoning begins with a brief description to puffer poisoning and then elucidates the origin, toxicology, chemistry, and pharmacology of TTX. The identification methods of puffer species are also

presented. TTX is especially hazardous among marine toxins in the world, especially in Asian countries. TTX causes traditional poisoning incidents due to ingestion of toxic puffer in Japan, Taiwan, and China. Its poisoning incident continues to occur in spite of tendency of decrease yet. Effective medical treatment has not so far been established for puffer poisoning. Along this way, some attempts were made to prepare an antibody against experimental animals, with some promising results. Intoxification mechanism of TTX-bearing orgainsms such as puffer, gastropod, and so on was found to come from a food web, resulting in further discovery of them. Accordingly, the distribution was enlarged, though the number was limited. The structure of TTX was clearly elucidated in 1964. At present, it is demonstrated that TTX is composed of more than 10 components, which differ from each other in side chain as well as in specific toxicity. TTX has long been detected by the mouse bioassay method, which is simple but not specific for this toxin. Recently, instrumental analyses have been widely applied to various natural substances such as HPLC, GC–MS, and HPLC–MS. TTX blocks sodium channels distributed on the cell membrane, and inhibits sodium ion flow in and out of the cell, resulting in paralysis of the muscle concerned. Applying the mechanism of action, TTX is often used as a specific pharmacological tool in studying nerve/muscle physiology. Furthermore, to prevent from intoxification due to ingesting the toxic puffer products, the DNA and protein techniques have been ultilized to identify the puffer species.

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