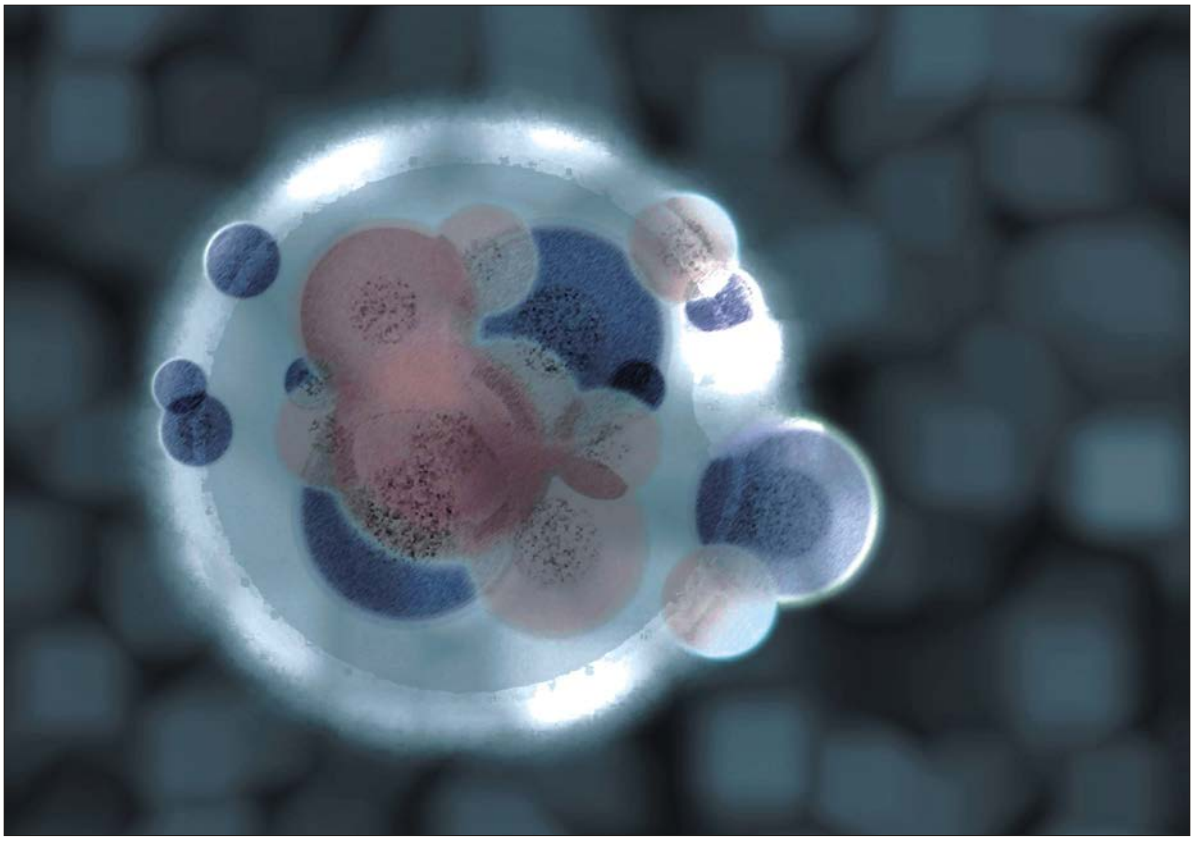

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 9 (5): 482-487, 2009
ISSN 1727-3048

Toxicity of Puffer Fishes (*Lagocephalus wheeleri* Abe, Tabeta and Kitahama, 1984 and *Lagocephalus sceleratus* Gmelin, 1789) from the East Coast Waters of Peninsular Malaysia

[1]K.D. Simon, [2]A.G. Mazlan and [1]Gires Usup
[1]Marine Science Programme, School of Environmental and Natural Resource Sciences,
Faculty of Science and Technology, Universiti Kebangsaan Malaysia,
43600 UKM Bangi, Selangor DE, Malaysia
[2]Marine Ecosystem Research Center, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor DE, Malaysia

Abstract: Toxicity analysis of the puffer fishes *Lagocephalus wheeleri* and *Lagocephalus sceleratus* from the East Coast Water of Peninsular Malaysia was carried out. The presence of tetrodotoxin (TTX) in fish tissue and cultures of bacteria isolated from the liver of the fish were determined. Detection of TTX was carried out by mouse bioassay, Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). The bacteria *Shewanella* sp. was isolated from the liver of *L. wheeleri* while, *Exiguobacterium* sp. and *Staphylococcus* sp. were isolated from the liver of *L. sceleratus*. Mouse bioassay showed that tissue extracts of *L. wheeleri* and culture supernatants of *Shewanella* sp. were positive for TTX. Tissue extracts of *L. sceleratus* and culture supernatants of *Exiguobacterium* sp. and *Staphylococcus* sp. exhibited non-lethal toxicity to mice but the symptoms were not typical of TTX poisoning. The symptoms in mice, coupled with TLC and HPLC analysis indicated that the toxic factor in *L. wheeleri* and *Shewanella* sp. was TTX. This study has confirmed the toxicity of the puffer *L. wheeleri* and some of this toxicity may be attributed to symbiotic bacteria.

Key words: Puffer fish, tetrodotoxin, toxin producing bacteria

INTRODUCTION

There are 185 species and 28 genera of puffer fish in the family Tetraodontidae (Oliveira *et al*., 2006). According to Sabrah *et al*. (2006) and Froese and Pauly (2007), these fishes typically occur in tropical seas and estuarine environments. Among the 185 species, *Lagocephalus wheeleri* and *Lagocephalus sceleratus* are the two most common puffer fish species in Malaysia and the former are eaten by some locals. These have resulted in several poisoning cases including a few fatalities (Kan *et al*., 1987). Many puffer fish species contain the neurotoxin tetrodotoxin (TTX) which is one of the most potent marine toxin (Yang *et al*., 1995). *Fugu vermicularis vermicularis* is a well known producer of TTX and anhydro-TTX (Jensen and Fenical, 1994; Noguchi *et al*., 1987; Sugita *et al*., 1987). Levels of TTX in puffer fishes may vary according to season, geographic location, sex and organ tissue (Noguchi *et al*., 1997; Nuñez-Vázquez *et al*., 2000; Yu and Yu, 2002). Most of the toxin is concentrated in the liver, gonads, intestines and skin of these fishes and probably contributed to approximately 60% of fatal cases (Ellenhorn and Barceloux, 1988).

However, TTX is not restricted to puffer fish and is widely distributed among various animal classes such as the Californian newt *Tarichi torosa* (Mosher *et al*., 1964), the goby *Gobius criniger* (Noguchi and Hashimoto, 1973), *Atelopus* frogs (Kim *et al*., 1975), the gastropod mollusks *Charonia sauliae* (Narita *et al*., 1981) and *Babylonia japonica* (Noguchi *et al*., 1981; Yasumoto *et al*., 1981), the xanthid crab *Atergatis floridus* (Noguchi *et al*., 1983), the blue-ringed octopus *Octopus maculosus* (Sheumack *et al*., 1978), *Astropecten* starfishes (Maruyama *et al*., 1984, 1985; Noguchi *et al*., 1982), the frog shell *Tutufa lissostoma* (Noguchi *et al*., 1984) and the small gastropod mollusks *Zeuxis siquijorensis* (Narita *et al*., 1984) and *Niotha clathrata* (Jeon *et al*., 1984).

The occurrence of TTX in these various taxa suggested that the toxin could either be acquired through the food web or is the product of bacterial symbionts in these species (Kosuge *et al*., 1985; Matsui *et al*., 1989). Many studies have been carried out to determine the origin of TTX in marine animals (Lee *et al*., 2000). Results from one study showed that *Vibrio fischeri* isolated from the xanthid crab *Atergatis floridus* and *Vibrio alginolyticus* and *Shewanella* sp. isolated from

Corresponding Author: Mazlan Abd. Ghaffar, Marine Ecosystem Research Center, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor DE, Malaysia
Tel: +603-89213219 Fax: +603-89253357

the puffer fish were able to produce TTX in pure cultures (Noguchi *et al*., 2006). The exogenous origin of TTX in puffer fish was first proposed by Matsui *et al.* (1981) in feeding experiments with artificially bred larvae. Later studies suggested that all TTX-bearing organisms harbour symbiotic bacteria capable of producing TTX (Matsui *et al*., 1985; Mosher and Fuhrman, 1984) and was later confirmed by isolation of TTX-producing bacteria from various TTX-bearing animals.

Puffer fishes are very common in Malaysian water are often caught in large numbers by trawlers or line fishing. *Lagocephalus wheeleri* is consumed in some parts of the country and puffer fish poisoning is quite frequent (Kan *et al*., 1987; Kanchanapongkul, 2001; Zaki *et al*., 2005). However, very few studies have been conducted on the toxicity of these fishes and their symbiotic bacteria. In this study, we tested the toxicity of some fish specimens originating from the East Coast Water of Peninsular Malaysia and also the toxicity of pure bacteria cultures isolated from the fishes.

MATERIALS AND METHODS

Fish samples: A total of 232 puffer fish comprising *Lagocephalus wheeleri* (84) and *Lagocephalus sceleratus* (148), were collected in September 2006 from the East Coast Water of Peninsular Malaysia. Samples were collected using a standard bottom trawl net. Details of the trawling operations are shown in Table 1. After hauling, the catch was removed and sorted into species groups. The puffer fish specimens were dissected and the body, gonad and liver weight was recorded. The dissected parts were then pooled together for further analysis in the laboratory.

Symbiotic bacteria isolation and culture: Each pool of puffer fish organ was homogenized in a sterile blender and suspended in 5 mL of sterile seawater. Serial dilutions were made to a final concentration of 0.05 g tissue mL^{-1}. Isolation of bacteria was achieved by spreading 0.5 mL of the diluted samples on Marine Agar plates. The plates were incubated at 28°C for 5-14 days to permit bacterial growth. Pure colonies were obtained by the streak-plate method onto new Marine Agar plates. The pure cultures were subcultured for several times to ensure purity prior to bacterial identification by morphological, physiological and biochemical tests. The tests used included gram staining, API 20 E, as well as oxidase and catalase tests.

Bacteria culturing for toxicity test: Bacteria cultures for toxicity tests were grown in sterilize Ocean Research Institute (ORI) medium (Simidu and Tsukamoto, 1985). The medium contained (g L^{-1}) protease peptone No. 3 (Difco), 2 g; bacto-yeast extract (Difco), 2 g; phytone peptone (BBL), 1 g; sodium thiosulphate, 0.4 g; sodium sulphite, 1 g; iron citrate, 0.08 g; seawater, 750 g and made up to 1 L with distilled water and adjusted to pH 7.6. Cultures were grown in 500 mL volumes and incubated at 25°C for 10 days in darkness without aeration (Yasumoto *et al*., 1986).

Table 1: Sampling locations, number of trawls, duration, average speed and trawling ranges

Geographic location		No. of trawls	Duration	Average speed	Range
Start	End	(n)	(h)	(knot)	(km)
6°00'N, 102°47'E	6°05'N, 102°39'E	3-4	3	3.0	17-20
5°58'N, 102°44'E	6°01'N, 102°38'E	2-3	3	2.8	10-13
6°01'N, 102°38'E	6°02'N, 102°42'E	2-3	3	2.2	7-10
5°51'N, 102°47'E	5°50'N, 102°48'E	3-4	3	2.3	11-13

Isolation and purification of TTX from bacteria cultures: The broth culture was centrifuged at 3000 rpm for 15 min to obtain a cell-free supernatant. The supernatant was transferred into new centrifuge tubes. The supernatant was evaporated in reduced pressure to a volume of about 100 mL and was then mixed with an equal volume of water-washed activated charcoal under agitation and filtered through a Buchner funnel. The charcoal on the funnel was thoroughly washed with distilled water and the TTX adsorbed was eluted 3 times with 3 volumes of 1% acetic acid in 20% aqueous ethanol. The elute was evaporated using a rotary evaporator. After the volume was reduced to 5 mL the sample was lyophilized, followed by addition of 10 mL 0.03 M acetic acid. This sample was used for detection of TTX by HPLC following the methods of Yu *et al*. (2004).

Processing of the fish organs for toxicity tests: From the frozen samples, 5 g each of gonad and flesh obtained and placed into separate tube containing 5 mL 0.03 M acetic acid and mashed using an ultrasonicator probe on ice. The slurry was centrifuged at 6000 rpm for 5 min to obtain the supernatant. The collected supernatant was later used for TTX detection.

Mouse bioassay: The mice used for the bioassay were female pure-bred Wistar strain each weighing around 20 g. One milliliter of the bacteria and fish extracts were injected intraperitoneally. Two mice were used for each sample. Symptoms were noted and the death time was recorded at the last gasping breath of the mouse (Yu *et al*., 2004).

Thin-Layer Chromatography (TLC): Thin-Layer Chromatography (TLC) was performed according to (Hwang *et al*., 1989). Briefly, samples were spotted on 5×20 cm silica gel 60 F_{254} precoated plates (Merck) and eluted with two solvent systems, pyridine: ethyl acetate:

acetic acid: water (15:5:3:6) and 1-butanol:acetic acid: water (2:1:1). Toxins were visualized as a yellow fluorescent spot under UV (365 nm) after spraying the plate with 1% H_2O_2 and 10% KOH.

High-Performance Liquid Chromatography (HPLC): High-Performance Liquid Chromatography (HPLC) analysis was performed on a Shimadzu VP system with a Pickering post-column reaction system. Separation was performed using an Agilent Hipersil AA-ODS column (2.1 mm i.d. ×200 mm). The mobile phase was 2 mM sodium 1-heptanesulfonic acid and 1% methanol in 0.05 M potassium phosphate buffer (pH 7.0) at a flow rate of 1 mL min^{-1}. Sample injection volume was 10 μL. The post-column reagent was 4 N NaOH. The column temperature was kept at 37°C for all runs, while the post column temperature was set at 100°C. Detection wavelengths were set at 380 nm excitation and 505 nm emissions. All samples were analyzed twice (Hwang *et al*., 1989).

RESULTS

Three pure bacteria cultures were isolated from the livers of *L. wheeleri* and *L. sceleratus* and designated as isolates 1, 2 and 3. The morphological and biochemical characteristics of the three bacteria isolates are shown in Table 2. The results suggested that isolate 1 from *L. wheeleri* was probably a *Shewanella* species while isolates 2 and 3 were probably *Exiguobacterium* and *Staphylococcus* species, respectively.

Tissue extracts of *L. wheeleri* and culture supernatants of bacterial isolate 1 from the fish liver were determined to be toxic in the mouse bioassay. Positive symptoms were observed between 5-7 min after injection resulting in death of the mice. In contrast *L. sceleratus* tissue extracts and culture supernatants of bacteria isolated from the fish did not kill mice. Toxicity effects were present but the symptoms were not typical of TTX poisoning. Detailed observations from the mouse bioassays are shown in Table 3.

Using the pyridine: ethyl acetate-acetic acid: water as solvent system, spraying the plate with 1% H_2O_2 and 10% KOH revealed a single spot with an R_f value of 0.78 for both bacterial supernatant (sample 1/isolate 1) and tissue extracts (sample 2, flesh) of *L. wheeleri* (Fig. 1). In contrast, sample 3 (isolate 3, bacterial supernatant of *L. sceleratus*) did not exhibit any spots. When the solvent system was replaced by 1-butanol: acetic acid: water (2:1:1), spraying the plate with 1% H_2O_2 and 10% KOH

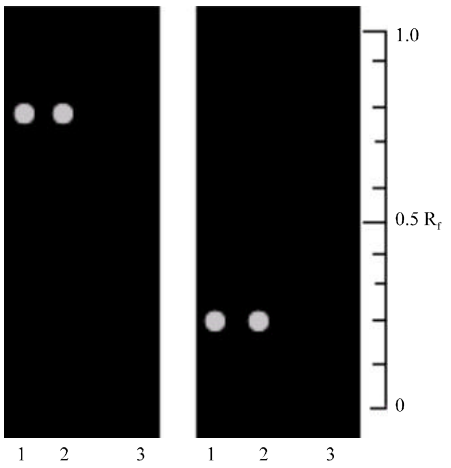

Fig. 1: Thin layer chromatography of the TTX fraction. (1: Sample 1, bacterial supernatant of *L. wheeler* (isolate 1), 2: Sample 2, tissue extract *L. wheeleri* (flesh), 3: Sample 3, bacterial supernatant of *L. sceleratus* (isolate 3)

Table 2: Morphological and biochemical characteristic of the three bacterial isolates

	Isolates		
Characteristics	1 (*L. wheeleri*)	2 (*L. sceleratus*)	3 (*L. sceleratus*)
Shape of colony	Straight	Straight	Circular
Elevation of colony	Flat	Flat	Convex
Colony margin	Undulate	Undulate	Entire
Colony pigmentation	Green	Orange	White
Cell shape	Short rod	Short rod	Round
Cell width (μm)	0.5-0.6	0.6-0.7	0.6-0.8
Cell length (μm)	1.3-1.4	1.4-1.6	1.2-1.3
Gram stain	Negative	Positive	Positive
Catalase activity	Positive	Positive	Positive
Oxidase activity	Positive	Positive	Positive
Putative bacteria	*Shewanella* sp.	*Exiguobacterium* sp.	*Staphylococcus* sp.

Table 3: Toxicities of *Lagocephalus wheeleri* and *Lagocephalus sceleratus* tissue extracts and isolated bacterial supernatants determined by mouse bioassay

		Symptoms						
Tissue and bacterial isolates	Injected volume (mL)	Fluffy and fat appearance	Sleepiness/ sluggishness	Twisted tail	Muscle contraction	Diarrhea	Gasping	Death
LS gonad	1	+	+	+	-	-	-	-
LW gonad	1	-	+	+	+	-	-	-
LS flesh	1	+	+	+	-	-	-	-
LW flesh (sample 2)	1	-	-	+	+	+	+	+ (5 min)
Isolate 1 (sample 1)	1	-	-	+	+	+	+	+ (6 min)
Isolate 2	1	+	+	+	-	-	-	-
Isolate 3 (sample 3)	1	+	+	+	-	-	-	-

+: Positively response, -: Negatively response, LW: *Lagocephalus wheeleri*, LS: *Lagocephalus sceleratus*, min: Minute

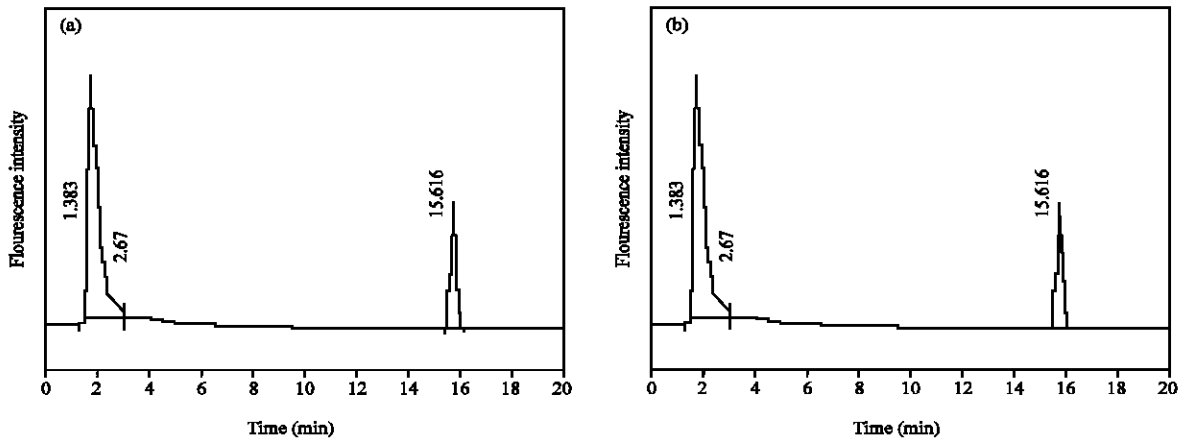

Fig. 2: *Lagocephalus wheeleri*. High-performance liquid chromatography HPLC of (a) sample 1 (isolate 1, bacterial supernatant) and (b) sample 2 (tissue extract, flesh)

revealed fluorescent spots for sample 1 and 2 apart from sample 3. In both cases for sample 1 and 2, the main spots, with an R_f value of 0.22, was similar to that of TTX (Fig. 1).

During HPLC analysis (Fig. 2a,b), only sample 1 and 2 displayed 2 peaks between the Retention times (Rt) -1.383 and 15.617 min, respectively. According to Shida *et al.* (1984), Hwang *et al.* (1989), Lee *et al.* (2000) and Jang and Yamashita (2006) synthesized of TTX and its compounds are optimal within the retention times of 14-20 min. Thus, the second peaks were a result of TTX-synthesis, whereas the first peaks were probably due to lipid, fat or other compounds.

DISCUSSION

Since 1986, the TTX-producing bacteria have been isolated form several phyla of TTX-bearing organisms in searching for the true origin of TTX in the marine and land animals. There are more than 60 indigenous species of puffer fish in Chinese seas (Zhu, 1998), but few studies have been conducted to investigate TTX-bearing animal and TTX-producing bacteria (Yu *et al.*, 2004). To present knowledge, this is the first study in which TTX-producing *Shewanella* sp. and non toxin producing *Exiguobacterium* sp. and *Staphylococcus* sp. were isolated from *L. wheeleri* and *L. sceleratus* in the East Coast Water of Peninsular Malaysia, which possess moderate to extreme toxic livers and flesh.

In the present study, *Shewanella* bacteria are dominant in the liver of *L. wheeleri* which has not been observed in *L. sceleratus*. This is not in agreement with earlier reports in which most TTX-producing strains are the members of genus *Vibrio*, *Pseudomonas*, *Alteromonas*, *Flavobacterium* and micrococcus (Miyazawa and Noguchi, 2001; Yu *et al.*, 2004). Nevertheless, Noguchi *et al.* (2006) confirmed that *Shewanella* sp. can produce TTX. Moreover, present results seemed to be partially in agreement with the results reported by Ellenhorn and Barceloux (1988) in terms of liver as a toxic organ, even though tissue extract of *L. sceleratus* and culture supernatant of *Exiguobacterium* sp. and *Staphylococcus* sp. exhibited some toxic effects not typical of TTX and did not kill mice.

TLC and HPLC analysis indicated that the toxic factor in *L. wheeleri* and *Shewanella* sp. was TTX based on the peaks between retention time ranges published earlier by Shida *et al.* (1984), Hwang *et al.* (1989), Lee *et al.* (2000) and Jang and Yamashita (2006). This positive result was only apparent from the tissue extract of flesh and bacterial supernatant from the liver of *L. wheeleri*, whereas *Exiguobacterium* sp. and *Staphylococcus* sp., which were isolated from the *L. sceleratus* liver did not show any positive results. In mouse bioassay it might be accountable for fluffy and fat appearance of the injected mice; however in HPLC tests it did not exhibit any peak between 14-20 min retention times. Noguchi *et al.* (1997), Nuñez-Vázquez *et al.* (2000) and Yu and Yu (2002) stated that in fish species concentration of TTX may vary due to season, geographic location, sex and organ tissue but in the present study only organ and tissue were taken into account.

In this study, we have shown that *L. wheeleri* specimens from the East Coast Waters of Peninsular Malaysia were toxic. We also showed that a bacteria isolate from the liver of the fish produced toxicity factor(s) that resembled the fish toxin characteristics. While it can not be definitely proven that the toxic factor was TTX, the symptoms, TLC and HPLC analysis strongly suggest that the toxin was TTX. Based on the results of this study it is recommended that further studies should be conducted

on puffer fishes and their associated bacteria for Malaysian waters in order to determine toxicity patterns related to season and geographic localities.

ACKNOWLEDGMENTS

This study was funded by the Malaysian government through UKM Science Fund Grant No. 04-01-02-SF0124. The authors would like to express their heartiest thanks to all dedicated laboratory technicians for their help in collecting fish used in this study. The technical assistance swiftly provided by Puan Hajah Mimi Nora, Cheng Kok Wah and Mei Ling in the microbial and HPLC analysis is deeply acknowledged. Special thanks to Rabi'a Ryklief at the Nelson Mandela Metropolitan University, South Africa for critical reading of the manuscript. We gratefully acknowledge the valuable comments of four anonymous reviewers. All field sampling and laboratory protocols followed and complied with the current laws of Malaysia. This study represents here was part of first author's MS work.

REFERENCES

Ellenhorn, M.J. and D.G. Barceloux, 1988. Medical Toxicology-Diagnosis and Treatment of Human Poisoning. Elsevier Science Publishing Co., Inc., New York.

Froese, R. and D. Pauly, 2007. Family Tetraodontidae-Puffers. FishBase, http://www.fishbase.org/Summary/FamilySummary.cfm?ID = 448.

Hwang, D.F., O. Arakawa, T. Saito, T. Noguchi, U. Simidu, K. Tsukamoto, Y. Shida and K. Hashimoto, 1989. Tetrodotoxin-producing bacteria from the blue-ringed octopus *Octopus maculosus*. Mar. Biol., 100: 327-332.

Jang, J. and Y.M. Yamashita, 2006. Distribution of tetrodotoxin, saxitoxin and their analogs among tissues of the puffer fish *Fugu pardalis*. Toxicon, 48: 980-987.

Jensen, P.R. and W. Fenical, 1994. Strategies for the discovery of secondary metabolites from marine bacteria. Annu. Rev. Microbiol., 48: 559-584.

Jeon, J.K., K. Miyazawa, T. Noguchi, H. Narita, K. Ito and K. Hasimoto, 1984. Occurrence of tetrodotoxin in a gastropod mollusk, araregai *Niotha clathrata*. Bull. Jpn. Soc. Sci. Fish., 50: 2099-2105.

Kan, S.K., M.K. Chan and P. David, 1987. Nine fatal cases of Puffer fish poisoning in Saban, Malaysia. Med. J. Malaysia., 42: 199-200.

Kanchanapongkul, J., 2001. Puffer fish poisoning: Clinical features and management experience in 25 cases. J. Med. Assoc. Thai., 84: 385-389.

Kim, Y.H., G.B. Brown and F.A. Fuhrman, 1975. Tetrodotoxin: Occurrence in atelopid frogs of Costa Rica. Science, 189: 151-153.

Kosuge, T., K. Tsuji, K. Hirai and T. Fukuyama, 1985. First evidence of toxin production by bacteria in a marine organism. Chem. Pharm. Bull., 33: 3059-3061.

Lee, M.J., D.Y. Jeong, W.S. Kim, H.D. Kim and C.H. Kim *et al*., 2000. A tetrodotoxin-producing *Vivrio* Strain, LM-1, from the puffer fish *Fugu vermicularis radatus*. Applied Environ. Microbiol., 66: 1698-1701.

Maruyama, J., T. Noguchi, J.K. Jeon, T. Harada and K. Hashimoto, 1984. Occurrence of tetrodotoxin in the starfish *Astropenten latespinisus*. Experientia, 40: 1395-1402.

Maruyama, J., T. Noguchi, H. Narita, M. Nara, J.K. Jeon, M. Otsuka and K. Hashimoto, 1985. Occurrence of tetrodotoxin in the starfish *Astropenten scoparius*. Agric. Biol. Chem., 49: 3069-3075.

Matsui, T., S. Hamada and S. Konosu, 1981. Difference in accumulation of puffer fish toxin and crystalline tetrodotoxin in the puffer fish, *Fugu rubripes rubripes*. Bull. Jpn. Soc. Sci. Fish., 47: 535-537.

Matsui, T., K. Yamamori, M. Chinone, S. Takatsuka, S. Sugiyama, H. Sato and C. Shimizu, 1985. Development of toxicity in cultured puffer fish kept with wild puffer fish? Development of toxicity in cultured Kusahugu (*Fugu niphobles*). Jpn. Soc. Sci. Fish., 1: 156-156.

Matsui, T., S. Taketsugu, K. Kodama, A. Ishil, K. Yamamori and C. Shimizu, 1989. Production of tetrodotoxin by the intestinal bacteria of a puffer fish, *Takifugu niphobies*. Nippon Suisan Gakkaishi., 55: 2199-2203.

Miyazawa, K. and T. Noguchi, 2001. Distribution and origin of tetrodotoxin. J. Toxicol. Toxicon Rev., 20: 11-33.

Mosher, H.S., F.A. Fuhrman, H.D. Buchwaid and H.G. Fischer, 1964. Tarichatoxin-tetrodotoxin: A potent neurotoxin. Science, 144: 1100-1110.

Mosher, H.S. and F.A. Fuhrman, 1984. Occurrence and Origin of Tetrodotoxin. In: Seafood Toxins, Ragelis, E.P. (Ed.). American Chemical Society, Washington, DC., pp: 333-344.

Narita, H., T. Noguchi, T.J. Maruyamai, Y. Ueda, K. Hoshimoto, Y. Watanabe and K. Hida, 1981. Occurrence of tetrodotoxin in a trumpet shell, boshubora *Charonia sauliae*. Bull. Japn. Soc. Sci. Fish., 47: 935-941.

Narita, H., T. Noguchi, J. Maruyama, M. Nara and K. Hashimoto, 1984. Occurrence of tetrodotoxin-associated substance in a gastropod, hanamushirogai *Zeuxis siquijorensis*. Bull. Jpn. Soc. Sci. Fish., 50: 85-89.

Noguchi, T. and Y. Hashimoto, 1973. Isolation of tetrodotoxin from a goby *Gobius criniger*. Toxicon, 11: 305-307.

Noguchi, T., J. Maruyama, Y. Ueda, K. Hashimoto and T. Harada, 1981. Occurrence of tetrodotoxin in the Japanese ivory shell *Babylonia japonica*. ull. Jpn. Soc. Sci. Fish., 478: 909-1013.

Noguchi, T., H. Narita, J. Maruyama and K. Hashimoto, 1982. Tetrodotoxin in the starfish *Astropenten polyacanthus*, in association with toxification of a trumpet shell, boshubora *Charonia sauliae*. Bull. Jpn. Soc. Sci. Fish., 48: 1173-1179.

Noguchi, T., A. Uzu, K. Koyama, J. Maruyama, Y. Nagashima and K. Hashimoto, 1983. Occurrence of tetrodotoxin as the major toxin in a xanthid crab, *Atergatis floridus*. Bull. Jpn. Soc. Sci. Fish., 49: 1887-1892.

Noguchi, T., J. Maruyama, H. Narita and K. Hashimoto, 1984. Occurrence of tetrodotoxin in the gastropod mollusk *Tutifa lissostoma* (frog shell). Toxicon, 22: 219-224.

Noguchi, T., D.F. Hwang, O. Arakawa, H. Sugita, Y. Deguchi, Y. Shida and K. Hashimoto, 1987. *Vibrio alginolyticus*, a tetrodotoxin-producing bacterium, in the intestines of the fish *Fugu vermicularis vermicularis*. Mar. Biol., 94: 625-630.

Noguchi, T., H. Akaeda and J.K. Jeon, 1997. Toxicity of a Puffer, *Takifugu vermicularis* 1. Toxicity of alive *Takifugu vermicularis* from Japan and Korea. J. Food Hyg. Soc. Japan, 38: 132-139.

Noguchi, T., O. Arakawa and T. Takatani, 2006. TTX accumulation in Pufferfish. Comp. Biochem. Physiol. Part D, 1: 145-152.

Nuñez-Vázquez, E.J., M. Yostu-Yamashita, A.P. Sierra-Beltrán, T. Yasumoto and J.L. Ochoa, 2000. Toxicities and distribution of tetrodotoxin in the tissues of puffer fish in the coast of Baja California Peninsula, Mexico. Toxicon, 38: 729-724.

Oliveira, J.S., R.S.C. Fernandes, C.A. Jr. Schwartz, C. Bloch, J.A.T. Melo Jr., O.R. Pires and J.C. Freitas, 2006. Toxicity and toxin identification in *Colomesus asellus*, an Amazonian (Brazil) fresh water puffer fish. Toxicon, 48: 55-63.

Sabrah, M.M., A.A. El-Ganainy and M.A. Zaky, 2006. Biology and toxicity of the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from the Gulf of Suez. Egypt. J. Aquat. Res., 32: 283-297.

Sheumack, D.D., M.E. Howden, I. Spence and R.J. Quinn, 1978. Maculotoxin: A neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. Science, 199: 188-191.

Shida, Y., K. Hashimoto and T. Noguchi, 1984. Occurrence of a tetrodotoxin-like compound as a minor toxin in the puffer fish, *Fugu flavidus*. Agric. Biol. Chem., 49: 2287-2291.

Simidu, V. and K. Tsukamoto, 1985. Habitat segregation and biochemical activities of marine members of the family vibryionaceae. Applied Environ. Microbiol., 180: 781-790.

Sugita, H., R. Ueda, T. Noguchi, O. Arakawa, K. Hashimoto and Y. Deguchi, 1987. Identification of a tetrodotoxin-producing bacterium isolated from the xanthid crab *Atergatis floridus*. Nippon Suisan Gakkaishi., 53: 1693-1693.

Yang, C.C., K.C. Han, T.J. Lin, W.J. Tsai and J.F. Deng, 1995. An outbreak of Tetrodoxin poisoning following gastropod mollusk consumption. Hum. Exp. Toxicol., 14: 446-450.

Yasumoto, T., Y. Oshima, M. Hosaka and H. Miyakoshi, 1981. Occurrence of tetrodotoxin in the ivory shell *Babylonia japonica* from Wakasa Bay. Bull. Jpn. Soc. Sci. Fish., 47: 909-914.

Yasumoto, T., D. Yasumura, M. Yotsu, T. Michishita and A. Endo, 1986. Bacterial production of tetrodotoxin and anhydrotetrodotoxin. Agric. Biol. Chem., 50: 793-795.

Yu, C.F. and P.H.F. Yu, 2002. The annual toxicological profiles of two common puffer fish: *Takifugu niphobles* Jordan and Synder and *Takifugu alboplumbeus* (Richardson), collected along Hong Kong coastal waters. Toxicon, 40: 313-316.

Yu, C.F., P.H.F. Yu, P.L. Chan, Q. Yan and P.K. Wong, 2004. Two novel species of tetrodotoxin- Producing bacteria isolated from toxin marine puffer fishes. Toxicon, 44: 641-647.

Zaki, M.A., A. El-Wahab and A. Mossa, 2005. Red sea puffer fish poisoning: Emergency diagonosis and management of human intoxication. Egypt. J. Aquat. Res., 31: 370-378.

Zhu, Q., 1998. A List of Marine Fishes in the China Seas. Xueyuan Press, Beijing.