表 2 *Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）における原因施設別の患者数によるリスクランキング—1988 年～2008 年—

順位	原因施設	標本数	平均値	上限値	下限値	F 値
1	学校・保育所等給食施設	66	87.7	1180.5	0.0	76.253**
2	仕出屋・弁当屋	9	33.8	267.7	−0.3	
3	旅館・ホテル	114	29.8	167.9	−0.3	
4	事業所給食施設	12	25.7	168.5	−0.3	
5	その他	60	23.1	175.1	−0.4	
6	調理実習施設（学校・公民館等）	56	21.4	94.1	−0.3	
7	販売店	3	21.2	114.3	−0.3	
8	寄宿舎・寮等給食施設	32	20.3	82.4	−0.3	
9	病院・老人ホーム等給食施設	6	13.4	92.5	−0.5	
10	飲食店（事業場・学校・病院は除く）	1158	9.0	42.6	−0.5	
11	家庭	40	3.5	14.8	−0.7	

**：P<0.01

表 3 *Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）における原因食品別の発生件数によるリスクランキング—1999～2008 年—

順位	原因食品	平均値	変動係数	上限値	下限値	F 値
1	鶏肉（生食品）	27.1	0.533	56.0	13.4	20.929**
2	食肉類（生食品）	7.7	0.943	22.2	5.4	
3	鶏肉（調理加工品）	7.2	0.720	17.6	3.7	
4	食肉類（調理加工品）	6.8	0.615	15.2	3.0	
5	使用水	0.5	1.414	1.9	−2.1	
6	弁当	0.5	1.054	1.6	−1.6	
7	複合調理品	0.3	2.250	1.6	−3.8	
8	和え物・サラダ	0.2	2.108	1.0	−3.8	
9	卵（調理加工品）	0.1	3.162	0.7	−6.0	
10	野菜・芋類（調理加工品）	0.1	3.162	0.7	−6.0	
11	めん・米飯・穀物類	0.1	3.162	0.7	−6.0	

**：P<0.01

品）の順であった。上限値の高い原因食品は，鶏肉（生食品），食肉類（生食品）の順であった。

発生件数の平均値が高く，上限値が高いもの，すなわち，発生頻度が特に高いものは，鶏肉（生食品），食肉類（生食品）であった。実際に，毎年，これら食品による発生件数は多く，また，年によっては著しく多くなることを示唆している。なお，鶏肉（生食品）は，下限値から毎年 13 件以上の発生が推測される。

表 4 は，Campy 食中毒の原因食品別の患者数の平均値，上限値および下限値を示した。

患者数の平均値が高い原因食品は，使用水，和え物・サラダ，弁当，複合調理品の順であった。上限値の高い原因食品は，使用水，和え物・サラダであった。

患者数の平均値が高く，上限値が高いもの，すなわち，健康被害の規模が特に大きいものは，使用水，和え物・サラダであった。これら食品の 1 事件ごとの患者数は多く，場合によっては患者数が著しく多くなることを示唆している。なお，使用水は明らかに水系感染を示すものであり，和え物・サラダを含め一度に提供する量や食数が多いので衛生管理の重要性が示唆された。

発生頻度が特に高い鶏肉（生食品）の患者数の順位は 8 位，食肉類（生食品）は 9 位と順位が低かった。これらは一度に提供される食数や量が少ないためであると考えられる。

以上，得られた原因施設および原因食品の疫学データによる計量値は，Campy 食中毒のリスクランキング，リスク管理を優先的に実施すべき施設や食品群の特定への寄与が期待できる。

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表 4 *Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）の原因食品別の患者数によるリスクランキング—1988 年～2008 年—

順位	原因食品	標本数	平均値	上限値	下限値	F 値
1	使用水	17	91.2	531.5	15.0	27.413 **
2	和え物・サラダ	4	47.7	427.6	4.5	
3	弁当	13	32.6	184.7	5.1	
4	複合調理品	5	32.4	213.0	4.2	
5	めん・米飯・穀物類	6	23.3	185.2	2.2	
6	鶏肉（調理加工品）	97	13.9	121.0	0.8	
7	食肉類（調理加工品）	84	13.0	67.8	1.9	
8	鶏肉（生食品）	304	9.1	42.6	1.3	
9	食肉類（生食品）	80	6.2	19.4	1.5	

** : P<0.01

3. 原因施設および原因食品の食中毒のリスクの高低によるグループ化

原因施設および原因食品は，発生件数や患者数の平均値を横軸，変動係数を縦軸とする散布図上に布置した。散布図は，さらに，これらの平均値を基準点として第Ⅰ象限から第Ⅳ象限の 4 つに分割した。各象限に布置された原因施設および原因食品のグループは次のように解釈される。

第Ⅳ象限は，基準点より平均値が高く変動係数が低い（バラツキが小さい）ので，食中毒のリスクが最も高いグループが布置される。

第Ⅰ象限は，第Ⅳ象限に比べバラツキが大きく食中毒のリスクが 2 番目に高いグループが布置される。

第Ⅲ象限は，第Ⅳ，第Ⅰ象限より食中毒のリスクが低いグループが布置される。

第Ⅱ象限は，食中毒のリスクが最も低いグループが布置される。

(1) 原因施設

図 2 は，原因施設の発生件数によるグループ化の成績である。基準点は，横軸 11.55 と縦軸 1.06 とした。

リスクランキングの順位が高かった飲食店は，第Ⅳ象限に布置された。飲食店は，最も発生頻度が高く，食中毒発生のリスクが最も高い施設であると考えられる。

第Ⅰ象限に布置された施設はなかった。

第Ⅲ象限の施設は，旅館・ホテル，その他，家庭，学校・保育所等給食施設，調理実習施設であった。毎年の発生件数は，第Ⅳ象限に布置された施設に比べて少ないことを示唆している。

第Ⅱ象限の施設は，事業所給食施設，寄宿舎・寮等給食施設，仕出屋・弁当屋，病院・老人ホーム等給食施設，販売店であった。これらは，第Ⅲ象限の施設に比べて毎年の発生件数に大きなバラツキがあることを示唆している。図 3 は，患者数による原因施設のグループ化の成績である。常用対数変換値による基準点は，横軸 1.46 と縦軸 0.30 とした。

リスクランキングの順位が高かった学校・保育所等給食施設，仕出屋・弁当屋，旅館・ホテルは第Ⅳ象限に布置された。これら施設は，一度に提供する食数が多い施設で，健康被害の規模が大きく，リスクが高い施設である。

第Ⅰ象限に布置された施設はなかった。

第Ⅲ象限に布置された施設は，事業所給食施設，調理実習施設，寄宿舎・寮等給食施設および販売店であった。これらの施設は，第Ⅳ象限に布置された施設に比べ 1 事例当たりの患者数が少ないことが示唆される。

第Ⅱ象限に布置された施設は，病院・老人ホーム等給食施設，飲食店，家庭およびその他であった。これらは，第Ⅲ象限に布置された施設に比べて患者数に大きなバラツキがあることを示唆している。以上のように，原因施設は，発生件数や患者数の平均値と変動係数，すなわち，リスクの高低により 4 つのグループに大別できた。

(2) 原因食品

図 4 は，原因食品の発生件数によるグループ化である。基準点は，横軸 4.6 と縦軸 1.7 とした。

リスクランキングの順位が高かった鶏肉（生食品），食肉類（生食品），鶏肉（調理加工品），食肉類（調理加工品）は，第Ⅳ象限に布置された。鶏肉（生食品）は，最も発生頻度が高く，食中毒発生のリスクが高い食品であると考えられる。

第Ⅰ象限に布置された食品はなかった。

第Ⅲ象限の食品は，使用水，弁当であった。毎年の発生件数は，第Ⅳ象限に布置された食品に比べて少ないこと

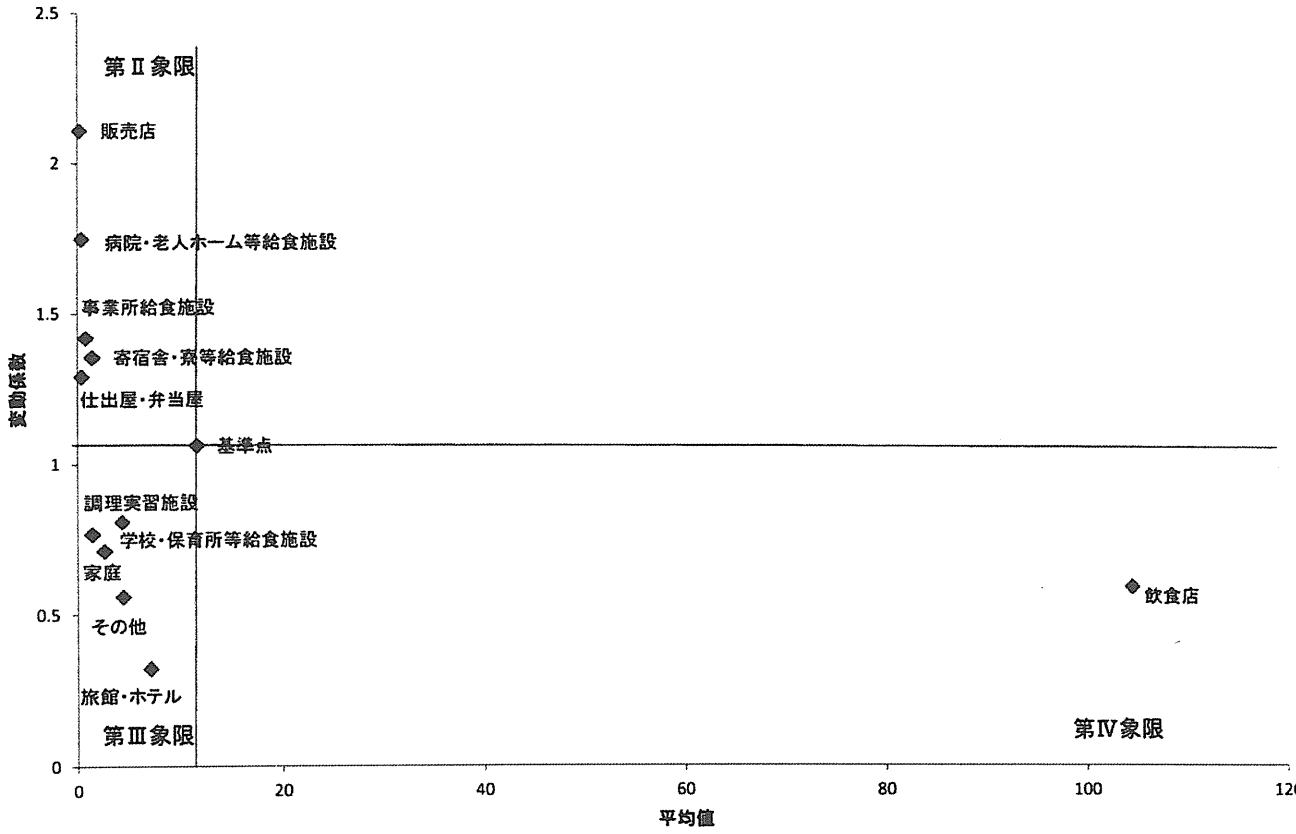

図 2 *Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）における原因施設別の発生件数の平均値と変動係数による散布図

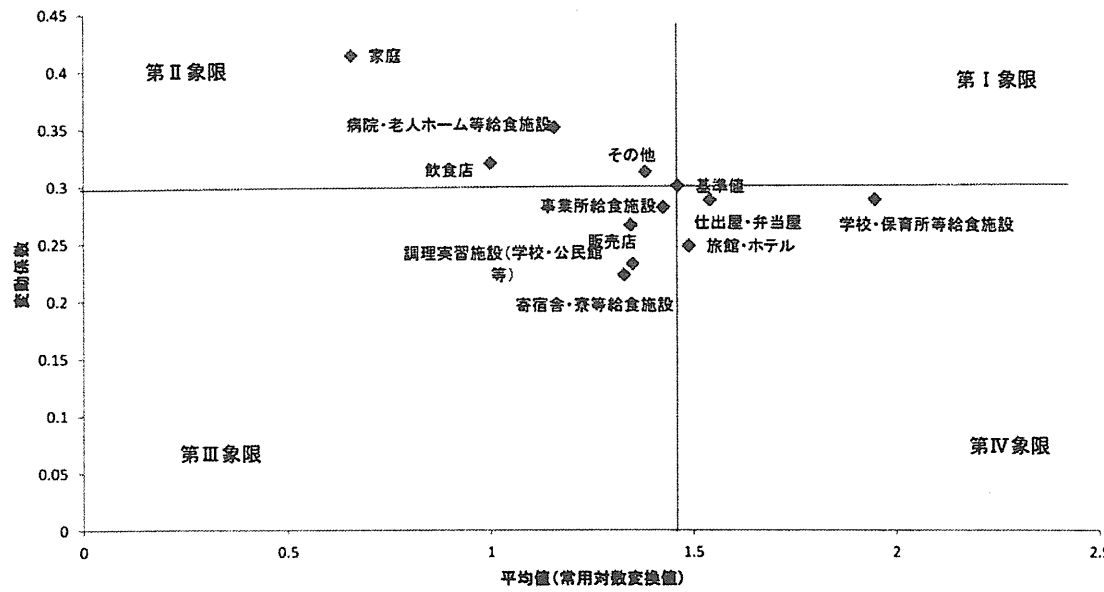

図 3 *Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）における原因施設別の患者数の平均値と変動係数による散布図

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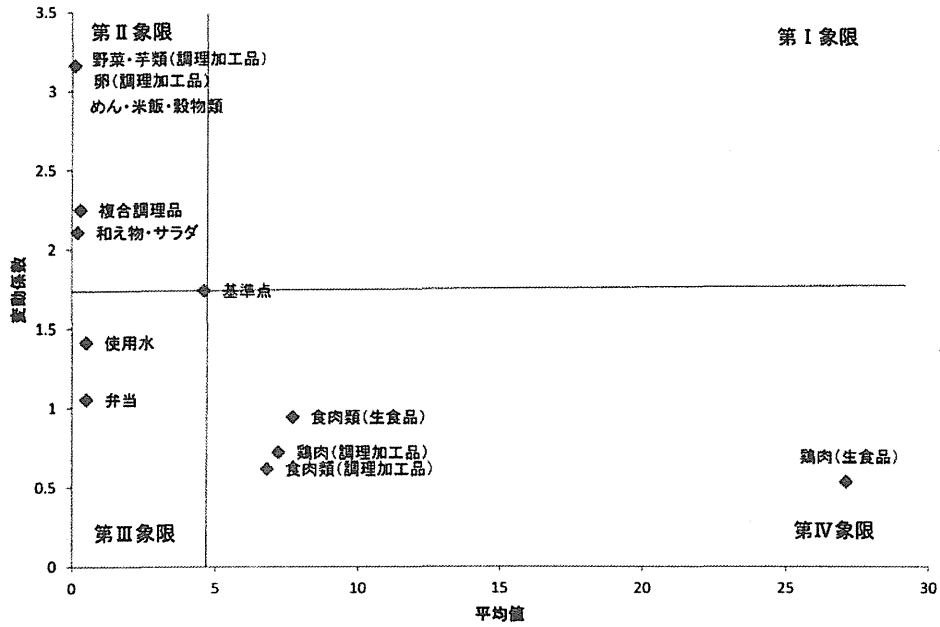

図 4　*Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）における原因食品別の発生件数の平均値と変動係数による散布図

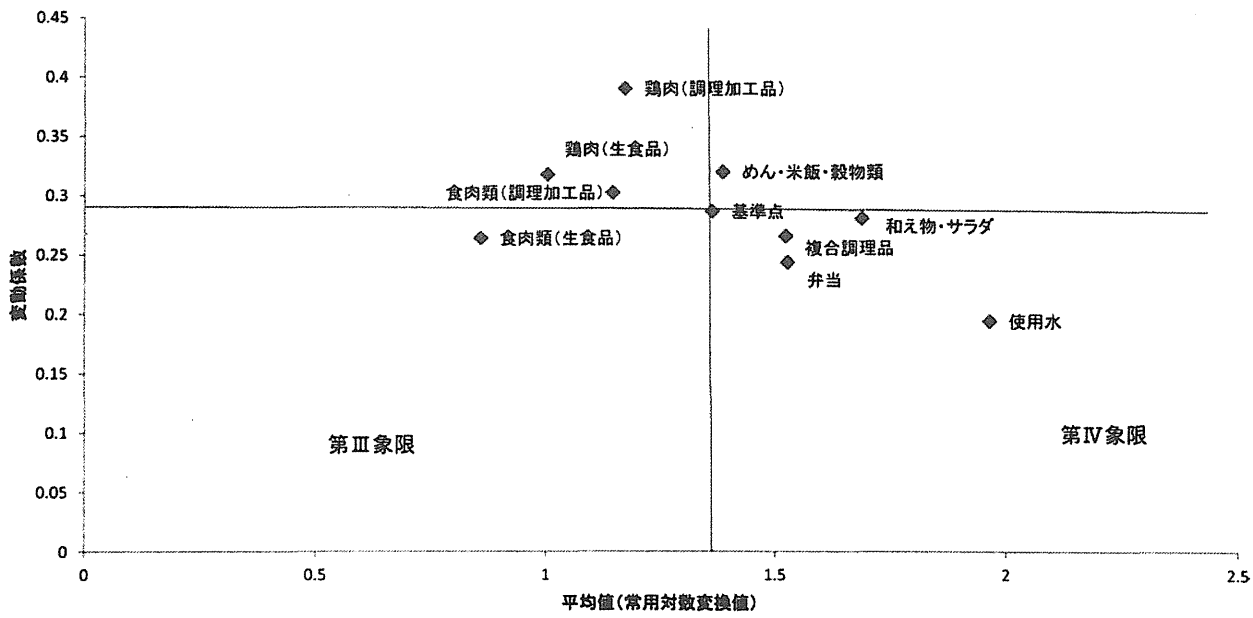

図 5　*Campylobacter* 食中毒（患者数 2 人以上，3,000 人未満）における原因食品別の患者数の平均値と変動係数の散布図

を示唆している。

第Ⅱ象限の食品は，和え物・サラダ，卵（調理加工品），野菜・芋類（調理加工品），めん・米飯・穀物類であった。これらは，第Ⅲ象限の施設に比べて毎年の発生件数に大きなバラツキがあることを示唆している。

図 5 は，患者数による原因食品のグループ化である。常用対数変換値による基準点は，横軸 1.36 と縦軸 0.29 とした。

リスクランキングの順位が高かった使用水，和え物・サラダ，弁当，複合調理食品は，第Ⅳ象限に布置された。これら食品は，一度に提供する食数が多い食品で，健康被害の規模が大きく，リスクが高い食品である。

第Ⅰ象限に布置された食品は，めん・米飯・穀物類であっ

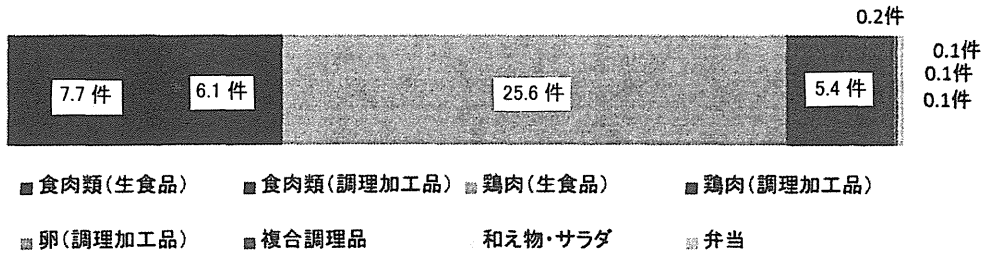

図 6 *Campylobacter* 食中毒（患者数2人以上，3,000人未満）で原因となった飲食店と原因食品の組合せの発生件数

た。

第Ⅲ象限に布置された食品は，食肉類（生食品）であった。第ⅠおよびⅣ象限に布置された食品に比べ，1事例当たりの患者数が少ないことから，一度に提供される食数が少ない食品であることが示唆される。

第Ⅱ象限に布置された食品は，鶏肉（調理加工品），食肉類（調理加工品），鶏肉（生食品）であった。これらは，第Ⅲ象限に布置された食品に比べて患者数に大きなバラツキがあることを示唆している。以上のように，原因食品は，発生件数や患者数の平均値と変動係数，すなわち，リスクの高低により，4つのグループに大別することができた。

4. 飲食店における原因食品

リスクランキングが第1位で，第IV象限に布置された飲食店における原因食品の発生件数は，図6に示すとおりであった。

最も発生件数が多かった原因食品は，鶏肉（生食品）（25.6件）であった。主な原因食品の割合は，鶏肉（生食品）（56.5%），食肉類（生食品）（17.0%），食肉類（調理加工品）（13.5%），鶏肉（調理加工品）（11.9%）で，鶏肉（生食品）および食肉類（生食品）が73.5%を占めていた。前述の東京都の報告では，生食あるいは半生食を原因食品とするものが約70%であり[1]，ほぼ同様の結果が得られた。

飲食店において，これら生食品を客に提供する際の対策が重要性であることが示唆された。同時に本研究により得られた数値は，リスクランキング，リスク管理目標値および評価基準値の設定に活用できるなど，リスク管理，さらにはリスクコミュニケーションへの一助となると示唆された。

結　論

1. Campy食中毒の発生件数の年次推移は，緩やかに上昇していたが，1999年以降急激な上昇傾向を示し，中心線（94.6件）を上回り，2008年，上方限界線（257.0件）を超えた。1999年以前と以降の発生件数の間には，危険率1%で有意差が認められた。

2. リスク管理において優先すべき，すなわち，健康被害の頻度が高い施設は，飲食店（104.5件），旅館・ホテル（7.0件），その他（4.3件），調理実習施設（学校・公民館等）（4.2件），家庭（2.5件）の順で，飲食店が最も頻度の高いグループであった。健康被害の規模の大きい施設は，学校・保育所等給食施設（87.7人），仕出屋・弁当屋（33.8人），旅館・ホテル（29.8人）の順で，これらが最も規模の大きいグループであった。

3. 健康被害の頻度が高い食品は，鶏肉（生食品）（27.1件），食肉類（生食品）（7.7件），鶏肉（調理加工品）（7.2件），食肉類（調理加工品）（6.8件）の順で，これらが最も頻度の高いグループであった。健康被害の規模の大きい食品は，使用水（91.2人），和え物・サラダ（47.7人），弁当（32.6人），複合調理品（32.4人）の順で，これらが最も規模が大きいグループであった。

4. 健康被害の頻度が最も高い飲食店において最も発生件数が多かった原因食品は，鶏肉（生食品）（25.6件）であり，以下，食肉類（生食品），食肉類（調理加工品），鶏肉（調理加工品）の順で，鶏肉（生食品）および食肉類（生食品）が73.5%を占めていた。

謝　辞

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ANALYSIS OF RISK MANAGEMENT REPORTS IN FOOD SERVICE PRACTICAL TRAINING COURSE

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ABSTRACT

The aim of this research is to identify risk factors in food services in large organizations and institutions so that food related illnesses or accidents can be prevented. Delineating the risk factors in food service process can reduce the occurrence of accidents such as food poisoning. In addition, identifiable risks can be incorporated in training the dietitians. Third year students enrolled in a Food Service Practical Training Course execute various tasks of servicing food at hospitals, nursing homes, schools, and large company cafeterias. In a simulated lab with machines and tools that are used in real settings, students prepare lunch according to the Good Hygienic Manual1). Students are assigned to serving food each week and generate risk-factor reports. A total of 131 reports were submitted during the five-week-course in the first semester in 2012. The data showed that 58.1% of the risks were found in seven areas: receiving ingredients, food storage, food preparation, cold cooking, heating food, storage of cooked food, and serving food. In these seven areas, the highest risk areas were food preparation and serving food. In food preparation, students dropped pieces of gloves in the food and misused aprons; and in serving food, the serving staff found small pieces of glove in served dishes, forgot use an apron, and they washed plates and serving utensils before all dishes were served. These risks occurred due to students' lack of understanding about hazard analysis and critical control points. Further analysis on larger sample data is required to complete the scale of risk factors in implementing HACCP in food service.

Keywords: risk management, food service, Good Hygienic Manual

INTRODUCTION

Dietitians and Nutritionists in Japan typically work in large institutions such as schools, hospitals, and nursing homes. They provide balanced nutrition to students and patients, and they are in charge of Food Hygienic Control. To do so, dietitians take the leadership role while working with other food service staff to make sure the cooking staff complies with hygienic regulations for handling food.

Students enrolled in the department of Nutrition expect to work as dietitians upon graduation. During their coursework, these future dietitians receive trainings about food hygienic practice. In Food Service Practicum I and II, the third year students are trained to provide proper diet to large number of people with specific nutritional needs. Areas of training include 1) completing each assigned compartmentalized job involved in food service management; 2) assimilating the role of dietitians and food service operating manager. In 15 classes, the students are assigned to serve five lunches. They are assigned to a team of 8 members and are asked to cook sixty servings of food. On the day the lunch is served, two teams offer different menus and serve 120 servings. Another team of eight students are assigned to work as serving staff. This research was conducted to identify areas of failures in Good Hygienic Practices (GHP) in food services at large organizations and institutions to prevent occurrence of food related illnesses such as food poisoning. Students prepare the lunch according to the GHP manual following the HACCP regulations for large-scale food preparation establishments. They are supposed to learn why such failures occur and how prevent them through the course.

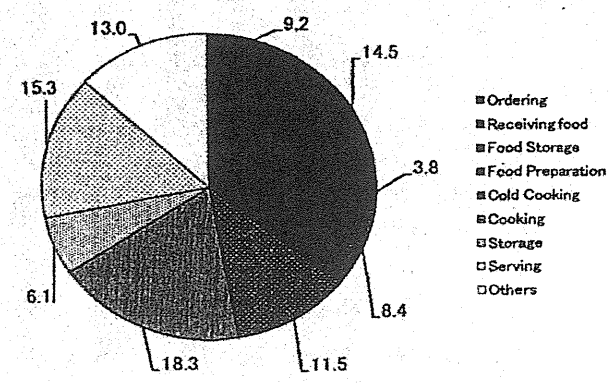

Fig.1. Failures in 7 areas (%). (n=131)

METHODS

For this research, risk management reports students submitted were analyzed. These reports identified the types, time of occurrence, and the reasons for the failures that happened during food service training. Students reported failures that are potential cause of accidents before and after each practicum session. The risk management reports contained seven areas (processes) of cooking lunch which are: (1) Receiving food, (2) Food storage, (3) Food preparation, (4) Cold cooking, (5) Cooking, (6) Storage of cooked food, and (7) Serving food.

RESULTS

In the first semester of 2012, 131 reports werecollected. The findings from these reports showed that the areas of failures were related to hygiene (49.6%) among the seven areas (Fig.1).

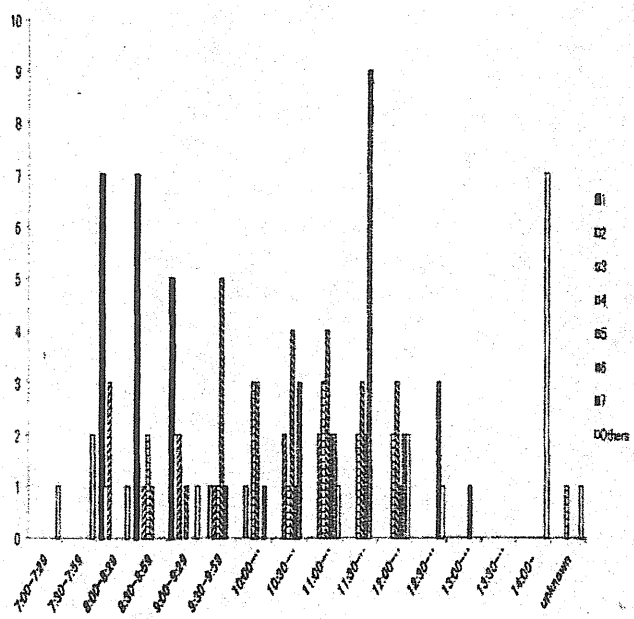

Fig. 2. The frequency of the failure tends to occur. (n=119).

The frequency of the failure tends to occur mostly in the early mornings and immediately before serving lunch (Fig.2). The most failures occurred from 7:30 to 8:59 a.m. and from11:30 to 11:59 a.m. In the detailed analysis of failures in each step of cooking, 19 incidents occurred during receiving food, and among these incidents, 36.8 % was from delivery, and 26.3% was from preparing and making sample dish (Fig.3). 5 incidents occurred during food storage, 40% of them was from leaving food out, another 40% of them was from using wrong container, and 20% of them was dropping food (Fig.4). 11 incidents occurred during food preparation,36.4% of them was from forgetting to wear aprons, 27.3% of them was from contamination of food, 18.2% of them was from operation equipment, such as not adjusting use of equipment, and 18.2 of them was from mishandling food (Fig. 5). 15 incidents occurred during, 26.7%of them was from not following recipes. Each 13.3 of them was from operating equipment, dropping food, equipment trouble. Each 6.7%of them was from broken gloves, misusage of gloves and excess of food (Fig. 6).

26 incidents occurred during cooking, 42.3% of them was not following recipes. 15.4% of them was from operating equipment, each 7.7 of them was from forgetting to wear aprons, forgetting to measure amount of meal, forgetting to put on gloves (Fig.7). 8 incidents occurred during food storage, each 25.0% of them was leaving food out and operating equipment. Each 12.5 of them was from forgetting to wrap food, dropping food, contamination of food and forgetting to measure the temperature of food (Fig. 8).

21 items incidents occurred during serving, 23.8% of them was from shortage of food. Each 14.3% of them was from forgetting to wear aprons and contamination of food. Each 9.5% of them was from wrong timing for serving meal, decoration, wrong amount serving, shortage of plate. Each 4.8% of them was from forgetting to put on gloves and washing containers during cooking (Fig. 9). 17 incidents classified to other situations, each 17.6% of them was from preparation, forgetting to change shoes and

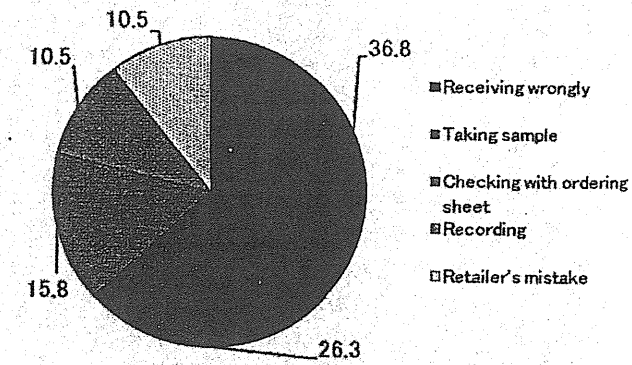

Fig. 3. Receiving food (%).(n=19)

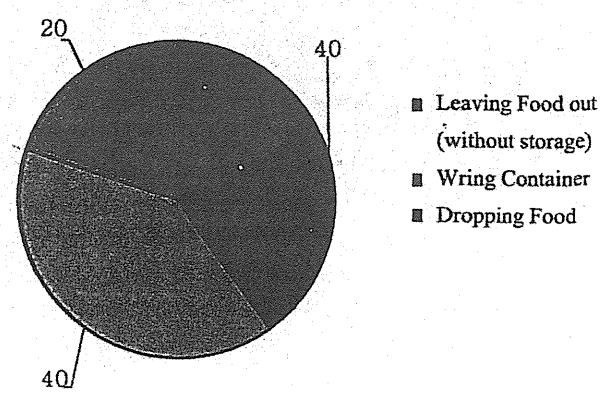

Fig. 4. Food Storage (%). (n=5)

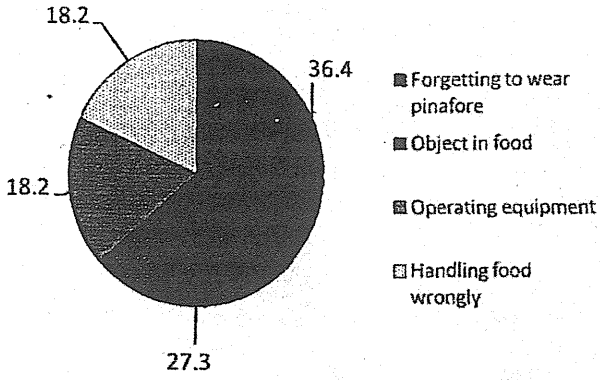

Fig. 5. Food Preparation (%).(n=11)

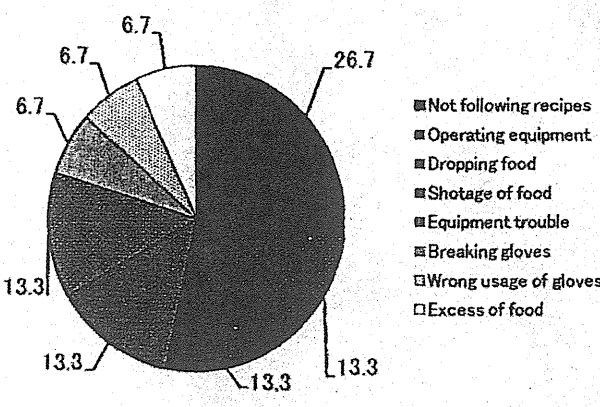

Fig. 6. Cold cooking (%).(n=15)

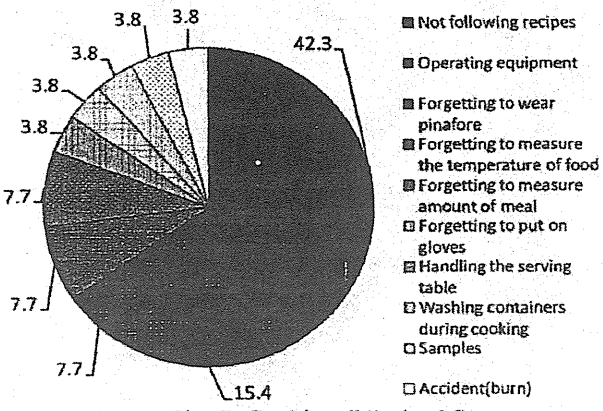

Fig. 7. Cooking (%). (n=26)

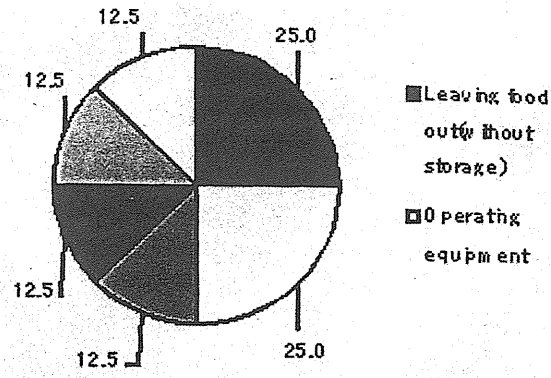

Fig. 8. Food storage (%). (n=8)

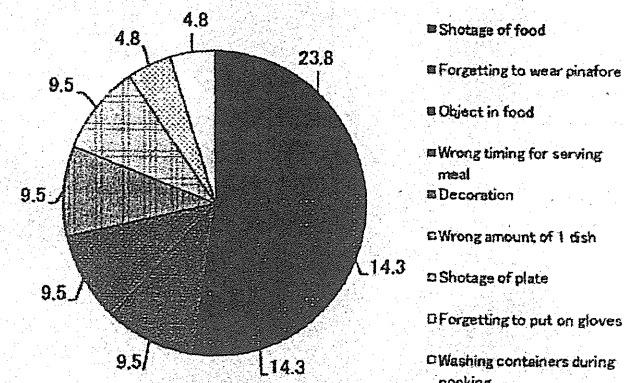

Fig. 9. Serving (%). (n=21)

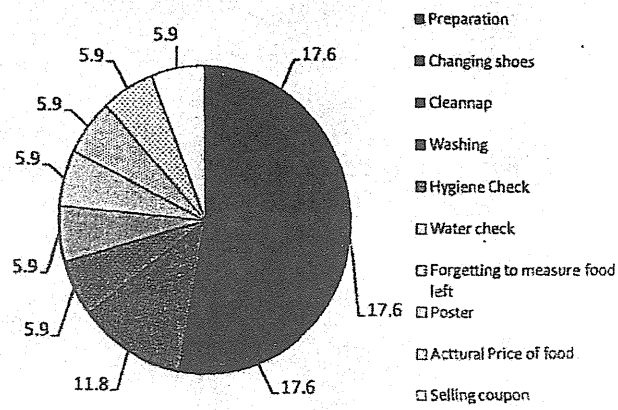

Fig. 10. Others (%). (n=17)

clean up. 11.8%of them was from washing. Each 5.9%of them was from hygiene check, water check, forgetting to measure left over, making poster, expenses for food and selling coupons.

DISCUSSION

The most failures had two peaks, one was from the preparation time such as from 7:30 and the other peak was just before the serving time. Since this, it is understood that students are not accustomed to handling a large amount of food which once were delivered and students had to check the amount of them as ordered and prepare for cooking, and prepare many plates for serving. From fig.4, it is difficult to make food storage properly, because they reported " I was too busy to storage food which we did not use soon". They need to make some schedules for food storage before serving.

From Fig.5, they tended to miss following the Good Hygienic Manual, such as wearing aprons and leading to contamination of food.

From Fig.6 and Fig.7, it was found the most failures occurred because not following recipes properly. Even though students exercise trials before the actual lunch services, it may be difficult to master seasoning large amount of food. From Fig.8, after cooking, if some dishes are still need to be storage, however, they did not notice that. From Fig.9, through the services, sometimes food shortage happened. It is difficult to estimate the amount of food when servings. From Fig.10, many cases were seemed, regarding forgetting to change shoes, it may be difficult to move from contamination areas to non-contamination area once the lunch service practice is on-going.

CONCLUSION

Many failures were seemed in the practice and most of them were seemed to come from understanding GHP and the lack of simulation. Some measures for future direction to reduce failures in GHP among students:

1. Increase instructional time for students to learn the Good Hygiene Manual.
2. Improve student retention rate through audio-visual training.
3. Simulation practice.
4. Visit other institutions, such as in schools, hospitals and nursing homes.

Understanding GHP through the audio- visual training and real simulation practice can be working for preventing failures.

REFERENCES

1] Ministry of Health, Labor and Welfare (1996). Good Hygienic Manual for large scale food preparation establishments.

Original Paper

Tetrodotoxin Poisoning Due to Smooth-backed Blowfish *Lagocephalus inermis* and Toxicity of *L. inermis* Caught off the Kyushu Coast, Japan

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Food poisoning due to ingestion of a puffer fish occurred in Nagasaki Prefecture, Japan, in October 2008, causing neurotoxic symptoms similar to those of tetrodotoxin (TTX) poisoning. In the present study, we identified the species, toxicity, and toxins using the remaining samples of the causative puffer fish. The puffer fish was identified as smooth-backed blowfish *Lagocephalus inermis* by nucleotide sequence analysis of the 16S rRNA and cytochrome *b* gene fragments of muscle mitochondrial DNA. The residual liver sample showed toxicity as high as 1,230 mouse unit (MU)/g by bioassay and TTX was detected by liquid chromatography/mass spectrometry analysis. We therefore concluded that the food poisoning was due to TTX caused by consumption of the toxic liver of *L. inermis*. This is the first report that the liver of *L. inermis* caught in Japanese waters is strongly toxic, with levels exceeding 1,000 MU/g. In this context, we re-examined the toxicity of *L. inermis* collected off the coast of Japan. Of 13 specimens assayed, 12 were toxic, although the toxicity varied markedly among individuals and tissues. Because the intestine and ovary of *L. inermis* have been considered non-toxic, it is particularly noteworthy that these organs were determined to be toxic, with a maximum toxicity of 43.6 MU/g and 10.0 MU/g, respectively. Furthermore, kidney, gallbladder, and spleen, whose toxicity has been unknown, were frequently found to be weakly toxic with levels ranging from 10 to 99 MU/g. Therefore, further study is needed to re-examine the toxicity of smooth-backed blowfish *L. inermis* in the coastal waters of Japan.

Key words: puffer fish poisoning; smooth-backed blowfish *Lagocephalus inermis*; puffer fish toxin; tetrodotoxin; toxicity; DNA species identification

Introduction

Food poisoning due to ingestion of a puffer fish occurred in Nagasaki Prefecture, Japan, in October 2008. According to a notice from Nagasaki City*1, three adults (two men and one woman) ate a puffer fish which had been purchased at a market in Nagasaki city and cooked as "*nitsuke*" (stewed in soy broth) of muscle, skin and liver or eaten as "*sashimi*". Two hours after ingestion, one man experienced neurotoxic symptoms, including numbness of the limbs, difficulty in walking and vomiting, similar to those of tetrodotoxin (TTX) poisoning.

The causative puffer fish sample is shown in Fig. 1 (the photograph was taken by the patient before cooking). The fish appears to be smooth-backed blowfish *Lagocephalus inermis* from its morphological appearance. Smooth-backed blowfish are often found in waters around southern Japan, as well as in tropical marine waters of the East China Sea, the South China Sea and the Indo-Pacific. This fish is regarded as an edible puffer fish species in Japan*2, because muscle, skin and testis are non-toxic although the liver is highly toxic[1), 2)]. However, it is reported that smooth-backed blowfish *L.*

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*1 Public Welfare and Health Department, Nagasaki City: Nagasaki City News Release (25th October, 2008), "An outbreak of food poisoning".

*2 Ministry of Health and Welfare, Japan: Director's Notice 59 (December 2nd, 1983), "To establish food hygiene of puffer fish".

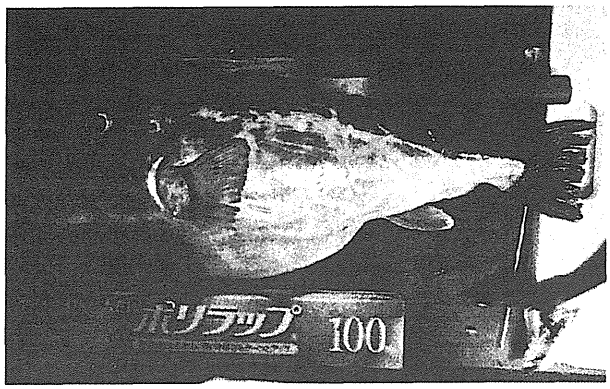


Fig. 1. Puffer fish sample which caused food poisoning in Nagasaki Prefecture, Japan in October 2008

inermis caught in Taiwan contained higher levels of toxins. Harada detected toxicity of as much as 22 mouse units (MU)/g in muscle of *L. inermis*[3]. In addition, Hwang *et al.* screened for toxicity of 25 species of puffer fish in Taiwan and demonstrated that not only liver, but also skin and testis from *L. inermis* specimens showed higher toxicity than those in Japan[4].

Recently, unusual appearance of tropical fish species, including puffer fish, off the Japanese coasts and sporadic food poisoning cases due to ingestion of these fish have been reported, possibly as a result of global warming[5]. In 2008 and 2009, puffer fish poisoning incidents involving green toadfish *Lagocephalus lunaris* occurred in western Japan[6]. These circumstances warrant increased awareness of the possibility of puffer fish poisoning. In this study, we identified the causative fish species of the puffer fish poisoning in Nagasaki Prefecture, Japan, in 2008 by a PCR amplification method and the responsible toxic principle by liquid chromatography/mass spectrometry analysis, and we re-examined the toxicity of smooth-backed blowfish *L. inermis* collected off the coast of Japan.

Materials and Methods

Materials

The uncooked remaining samples of muscle, skin and liver from the puffer fish responsible for the food poisoning in Nagasaki Prefecture in October 2008 were obtained and analyzed for toxicity, toxins and species identification.

For re-examination of the toxicity of *L. inermis*, thirteen specimens of smooth-backed blowfish *L. inermis* were caught off the Miyazaki coast, Japan, from 2001 to 2010. They were immediately frozen, transported to the Laboratory of Tokyo University of Marine Science and Technology, and stored at −30℃ until use.

Assay of toxicity

The leftover muscle, skin and liver of the causative puffer fish were subjected to toxicity testing as described below.

Other specimens caught off the Miyazaki coast were also assayed for toxicity. They were dissected into muscle, skin, liver, intestine, kidney, gallbladder, spleen and gonad (ovary or testis) after having been partially thawed. The tissue samples were minced with scissors or a scalpel and homogenized with 0.1% acetic acid. TTX was extracted by heating in a boiling water bath for 10 min according to the official guidance of the Japan Food Hygiene Association[7]. The toxicity of each sample was measured by bioassay using four-week-old male ddY strain mice weighing 20 g, following the above official guideline[7]. The toxicity in the mouse bioassay was expressed as mouse unit (MU) where one MU is defined as the amount of toxin that kills a mouse in 30 min after intraperitoneal injection. All the animal experiments were performed in compliance with the fundamental guidelines for proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, and approved by the animal experiment committee in Tokyo University of Marine Science and Technology or by the corresponding committee in Nagasaki University.

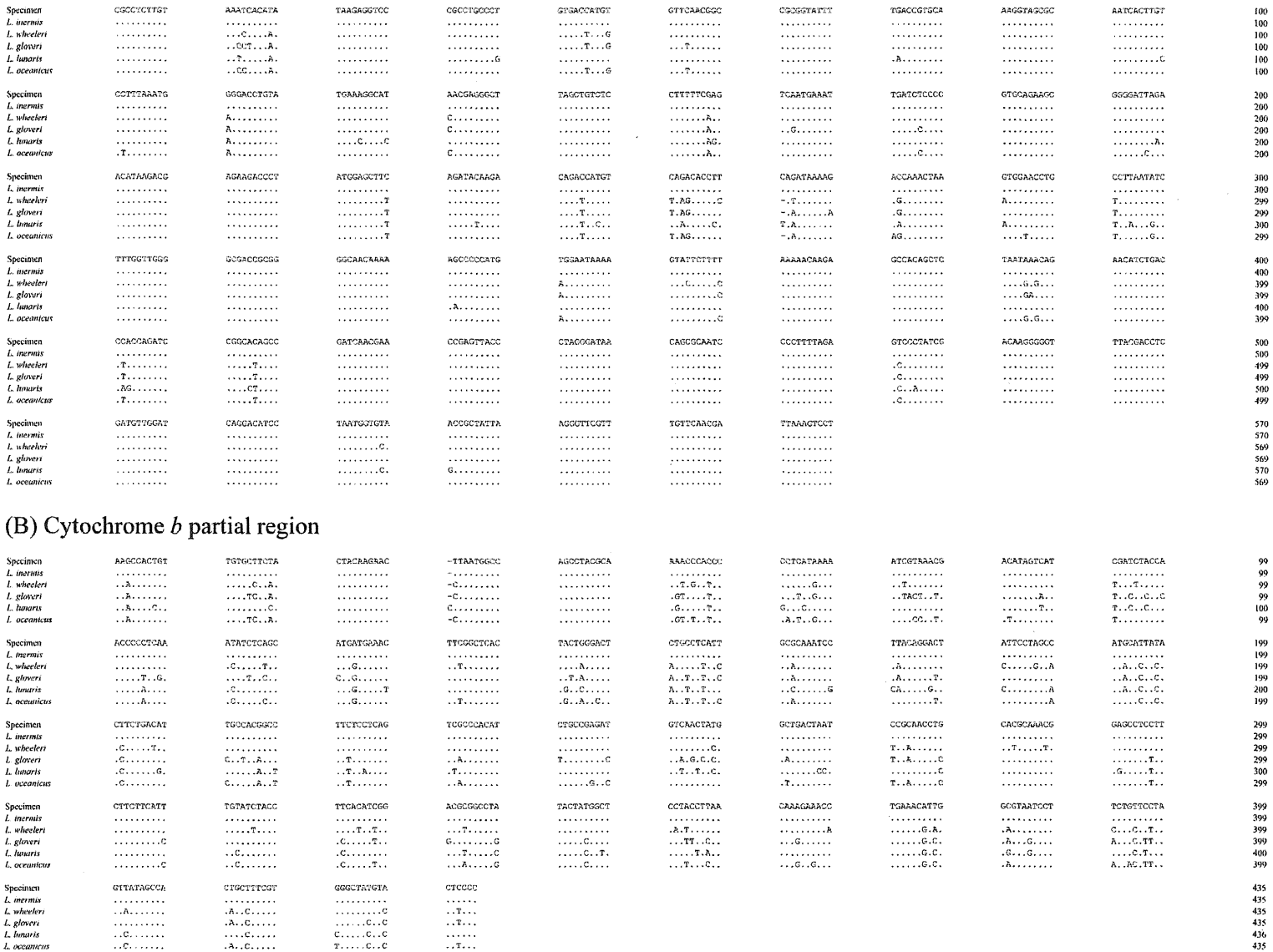
Toxin analysis

Aliquots of the leftover tissue extracts were filtered with a membrane filter (DISMIC-25CS, 0.45 μm, ADVANTEC, Japan) and ultrafiltered through a USY-1 (MWCO 10,000, ADVANTEC). The resulting filtrate was subjected to liquid chromatography/electron spray ionization-mass spectrometry (LC-ESI-MS) for analysis of the toxin profiles as previously reported[8]. Briefly, LC-ESI-MS was performed on an Alliance Zspray™ MS 2000 LC-ESI-MS system (Waters, Milford, MA, USA). The analytical column was a Mightysil RP-18GP (2.0× 250 mm, Cica-Reagent, Tokyo, Japan), maintained at 25℃. Elution was done with 30 mmol/L heptafluorobutyric acid in 1 mmol/L ammonium acetate (pH 5.0) at a flow rate of 0.20 mL/min. The eluate was introduced into the ion source block of the ESI-MS detector and ionized in the positive ion mode with a desolvation temperature of 350℃, ion source block temperature of 120℃, and cone voltage of 50 kV. TTX detection at m/z 320, corresponding to the protonated molecular ion $(M+H)^+$, was achieved using the selected ion recording mode.

DNA extraction and PCR amplification of mitochondrial 16S rRNA and cytochrome b gene fragments

Total cellular DNA was extracted from muscle of the causative fish with a DNeasy Blood & Tissue kit (Quiagen K.K., Tokyo, Japan) by the reported method[6), 9)]. In brief, 25 mg aliquots of muscle were mixed with 180 μL Buffer ATL and 40 μL proteinase K solution, incubated at 55℃ for 1 h, and centrifuged at 20,000×g for 15 min. The resulting supernatants were treated with 4 μL RNase A (100 mg/mL) for 2 min, followed by addition of 200 μL Buffer AL, incubation at 70℃ for 10 min, and then addition of 200 μL ethanol. DNA was purified with a DNeasy Mini Spin column. The preparations were applied to the column, which was washed with 500 μL Buffer AW1 and Buffer AW2 successively, and eluted

(A) 16S rRNA partial region



Specimen											
Specimen	CGCCYCTTGT	AAATCACATA	TAAGAGGTCC	CGCCTGCCCT	GTGACCATGT	GTTCAACGGC	CGCGGYATTT	TGACCGTGCA	AAGGTAGCGC	AATCACTTGT	100
L. inermis	....	....	....	....	....	....	....	....	....	....	100
L. wheeleri	....	...C...A..	....	....	.....T...G	....	....	....	....	....	100
L. gloveri	....	..CAT...A.	....	....	.....T...G	...T......	....	....	....	....	100
L. lunaris	....	..T.....A.	....	.........G	....	....	....	.A........	....	C	100
L. oceanicus	....	..CC...A.	....	....	.....T...G	...T......	....	....	....	....	100
Specimen	CGTTTAAATG	GGGACCTGTA	TGAAAGGCAT	AACGAGGGCT	TAGCTGTCTC	CTTTTTCGAG	TCAATGAAAT	TGATCTCCCC	GTGCAGAAGC	GGGGATTAGA	200
L. inermis	....	....	....	....	....	....	....	....	....	....	200
L. wheeleri	....	A.........	....	C.........	....	.......A..	....	....	....	....	200
L. gloveri	....	A.........	....	C.........	....	.......A..	..G.......	.....C....	....	....	200
L. lunaris	....	A.........	....C....C	....	....	.......AG.	....	....	....	.......A.	200
L. oceanicus	.T........	A.........	....	C.........	....	.......A..	....	.....C....	....	.....C...	200
Specimen	ACATAAGACG	AGAAGACCCT	ATGGAGCTTC	AGATACAAGA	CAGACCATGT	CAGACACCTT	CAGATAAAAG	ACCAAACTAA	GTGGAACCTG	CCTTAATATC	300
L. inermis	....	....	....	....	....	....	....	....	....	....	300
L. wheeleri	....	....	.........T	....	....T.....	T.AG....C	-.T.......	.G........	A.........	T.........	299
L. gloveri	....	....	.........T	....	....T.....	T.AG.....	-.A.....A	.G........	....	T.........	299
L. lunaris	....	....	.........T	.....T....	....T..C..	..A....C.	T.A.......	.A........	A.........	T..A...G..	300
L. oceanicus	....	....	.........T	....	....T.....	T.AG.....	-.A.......	AG........	....T.....	T......G..	299
Specimen	TTTGGTTGGG	GCGACCGCGG	GGCAACAAAA	AGCCCCCATG	TGGAATAAAA	GTATTCTTTT	AAAAACAAGA	GCCACAGCTC	TAATAAACAG	AACATCTGAC	400
L. inermis	....	....	....	....	....	....	....	....	....	....	400
L. wheeleri	....	....	....	....	A.........	...C.....C	....	....	....G.G...	....	399
L. gloveri	....	....	....	....	A.........	C	....	....	....GA....	....	399
L. lunaris	....	....	....	.A........	....	....	....	....	....	....	400
L. oceanicus	....	....	....	....	A.........	C	....	....	....G.G...	....	399
Specimen	CCAGCAGATC	CGGCACAGCC	GATCAACGAA	CCGAGTTACC	CTACGGATAA	CAGCGCAATC	CCCTTTTAGA	GTCCCTATCG	ACAAGGGGGT	TTACGACCTC	500
L. inermis	....	....	....	....	....	....	....	....	....	....	500
L. wheeleri	.T........	.....T....	....	....	....	....	....	.C........	....	....	499
L. gloveri	.T........	.....T....	....	....	....	....	....	.C........	....	....	499
L. lunaris	.AG.......	....CT....	....	....	....	....	....	.C..A.....	....	....	500
L. oceanicus	.T........	.....T....	....	....	....	....	....	.C........	....	....	499
Specimen	GATGTTGGAT	CAGGACATCC	TAATGGTGTA	ACCGCTATTA	AGGGTTCGTT	TGTTCAACGA	TTAAAGTCCT				570
L. inermis	....	....	....	....	....	....	....				570
L. wheeleri	....	....	........C.	....	....	....	....				569
L. gloveri	....	....	....	....	....	....	....				569
L. lunaris	....	....	........C.	G.........	....	....	....				570
L. oceanicus	....	....	....	....	....	....	....				569

(B) Cytochrome *b* partial region

Specimen											
Specimen	AAGCCACTGT	TGTGCTTCTA	CTACAAGAAC	-TTAATGGCC	AGCCTACGCA	AAACCCACCC	CCTCATAAAA	ATCGTAAACG	ACATAGTCAT	CGATCTACCA	99
L. inermis	....	....	....	-.........	....	....	....	....	....	....	99
L. wheeleri	..A.......	.....C..A.	....	-C........	....	..T.G..T..	.....G....	....	....	T...T.....	99
L. gloveri	..A.......	....TC..A.	....	-C........	....	.GT...T..	...T..G...	..TACT..T.	.....A..	T..C..C..C	99
L. lunaris	..A....C..	.........C.	....	C.........	....	.G.....T..	G...C.....	....	.....T..	T..C..C...	100
L. oceanicus	..A.......	....TC..A.	....	-C........	....	.GT.T..T..	.A.T..G...	....GC..T.	.T........	T.........	99
Specimen	ACCCCCTCAA	ATATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TACTGGGACT	CTGCCTCATT	GCGCAAATCC	TTACAGGACT	ATTCCTAGCC	ATGCATTATA	199
L. inermis	....	....	....	....	....	....	....	....	....	....	199
L. wheeleri	....	.C....T..	...G......	..T.......	....A.....	A....T..C	..A.......	.A........	C....G..A	..A..C..C.	199
L. gloveri	.....T..G.	....T..C..	C..G......	....	.T.A.....	A..T..T..C	..A.......	.A.....T.	....	..A..C..C.	199
L. lunaris	.....A....	.C........	...G.....T	....	.G..C.....	A..T..T...	..C......G	CA.....G..	C........A	..A..C..C.	200
L. oceanicus	.....A....	.C.....C..	...G......	..T.......	.G..A..C..	A..T..T..C	..A.......	.......T.	.........A	.....C..C.	199
Specimen	CTTCTGACAT	TGCCACGGCC	TTCTCCTCAG	TCGCCCACAT	CTGCCGAGAT	GTCAACTATG	GCTGACTAAT	CCGCAACCTG	CACGCAAACG	GAGCCTCCTT	299
L. inermis	....	....	....	....	....	....	....	....	....	....	299
L. wheeleri	.C.....T..	....	....	....	....	.......C.	....	T..A......	..T....T.	....	299
L. gloveri	.C........	C..T..A...	..T.......	..A.......	T........C	..A.G.C.C.	.A........	T..A.....C	....	.......T..	299
L. lunaris	.C......G.	......A..T	..T..A....	.T........	....	..T..T..C.	.......CC.	C	....	.G.....T..	300
L. oceanicus	.C........	C.....A..T	..T.......	..A.......	.....G..C	....	.T........	T..A.....C	....	.......T..	299
Specimen	CTTGTTCATT	TGTATCTACC	TTCACATCGG	ACGCGGCCTA	TACTATGGCT	CCTACCTTAA	CAAAGAAACC	TGAAACATTG	GCGTAATGCT	TCTGTTCCTA	399
L. inermis	....	....	....	....	....	....	....	....	....	....	399
L. wheeleri	....	.....T....	....T..T..	...T......	....	.A.T......	.........A	.....G.A.	.A........	C...C..T..	399
L. gloveri	C	....	.C.....T..	G........G	.....C....	...TT..C..	...G......	.....G.C.	.A...G....	A...C.TT..	399
L. lunaris	....	..C.......	.C........	...T.....C	.....C..T.	.....T.A..	....	.....G.C.	.G...G....	....C.T...	400
L. oceanicus	C	..C.......	.C.....T..	...A.....G	.....C....	...T...C..	...G..G...	.....G.C.	.A........	A..AC.TT..	399
Specimen	GTTATAGCCA	CTGCTTTCGT	GGGCTATGTA	CTCCCC							435
L. inermis	....	....	....	.....							435
L. wheeleri	..A.......	.A..C.....	C	..T...							435
L. gloveri	....	.A..C.....	.....C..C	..T...							435
L. lunaris	..C.......	....C.....	C.....C..C	.....							436
L. oceanicus	..C.......	.A..C.....	T.....C..C	..T...							435

Fig. 2. DNA sequence alignment of the amplified partial 16S rRNA (A) and cytochrome *b* (B) regions of mitochondrial DNA from muscle of the puffer fish specimen responsible for the food poisoning, along with those of *Lagocephalus inermis* (accession number G61747), *L. wheeleri* (accession number AP009538), *L. gloveri* (accession number CQ461748), *L. lunaris*[6], and *L. oceanicus* (unpublished data)

A dot (•) indicates identity with the 1st DNA sequence. A gap introduced into the sequences to optimize the alignment is represented by a dash (—).

with 200 μL AE Buffer.

Partial regions of the mitochondrial 16S rRNA (about 615 bp) and cytochrome *b* (about 487 bp) were amplified by conventional PCR using primer pairs 5′-CGCCTGTTTATCAAAAACAT-3′ (16Sar-L)/5′-CCGGTCTGAACTCAGATCACGT-3′ (16Sbr-H) for 16S rRNA and 5′-CAGGATTTTTAACCAGGACTAATGGCTTGAA-3′ (L14317Glu)/5′-CCCTCAGAATGATATTTGTCCTCA-3′ (H15149) for cytochrome *b*[10]. PCR was performed in 50 μL total volume of reaction buffer containing 4 μL of 2.5 mM dNTPs, 1.5 μL of 20 μM each primer, 0.4 μL of EXTaq™ DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), 5 μL of 10 ×EXbuffer (+$MgCl_2$), and 5 μL of extracted template DNA (1 μg). PCR was carried out with a thermal cycler PC-801 (Astec, Fukuoka, Japan). Amplifying conditions were 98℃ for 10 s for denaturing, 53℃ for 30 s for annealing, and 70℃ for 60 s for extension (30 cycles). The PCR products were analyzed by electrophoresis in a 2% agarose gel containing SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and observed with a luminescent image analyzer (LAS-4000 mini, FUJIFILM Cooperation, Tokyo, Japan).

DNA sequencing

After amplification, the PCR products were treated with ExoSAP-IT (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) to degrade unincorporated primers and dNTP. They were subsequently used as a DNA template for direct sequencing. DNA was sequenced with a BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA) and an ABI 3130 genetic analyzer (Applied Biosystems). To identify the puffer fish species, the sequences were searched against DNASIS Taxon V3.0 for Fugu (Hitachi Solutions Ltd., Tokyo, Japan) and the original database of puffer fish mitochondrial sequences in our laboratory at Tokyo University of Marine Science and Technology.

Results and Discussion

Figure 1 shows the puffer fish sample (about 40 cm body length) that caused food poisoning in Nagasaki Prefecture, Japan, in October 2008 and was apparently similar to smooth-backed blowfish (*Kanafugu* in Japanese). In the puffer fish poisoning incident, a part of the uncooked sample had been kept. For species identification, aliquots of the remaining muscle were subjected to DNA extraction and PCR amplification of 16S rRNA and cytochrome *b* gene fragments. DNA sequences of parts of mitochondrial 16S rRNA and cytochrome *b* from muscle of the puffer fish are shown in Fig. 2. The nucleotide sequences of mitochondrial 16S rRNA fragment (570 bp except primer region) and cytochrome *b* fragment (435 bp except primer region) of the puffer fish muscle were identical with those of *L. inermis*, but not with those of other puffer species of genus *Lagocephalus*, such as *L. wheeleri* (95.3 and 88.0% nucleotide sequence identity for 16S rRNA and cytochrome *b*, respectively), *L. gloveri* (94.9 and 82.1%), *L. lunaris* (94.2 and 85.1%) and *L. oceanicus* (94.6 and 83.2%). The results indicate that the puffer fish that caused the food poisoning is indistinguishable from smooth-backed blowfish *L. inermis*.

Toxicity of the remaining samples of liver, muscle and skin was measured by bioassay. The liver showed toxicity as high as 1,230 MU/g, while the muscle and skin showed toxicity as low as 3 MU/g. Figure 3 shows the LC/ESI-MS results for the extracts of liver, muscle and skin. In the selected ion mass chromatogram at *m/z* 320 for TTX ($C_{11}H_{17}O_8N_3$, 319.27 Da), the peak corresponding to TTX with a retention time of 6.86 min was detected in not only liver, but also muscle and skin. The TTX peak of the liver extract was estimated to be 944 MU/g, accounting for the major parts of the toxicity of the liver, although other TTX derivatives and paralytic shellfish poisoning toxins were not analyzed. The peaks of muscle and skin extracts were equivalent to only 1 MU/g. Therefore, it is most likely that the liver was the causative organ and TTX was the responsible toxic principle in this puffer fish poisoning. The toxin in muscle and skin samples might have bean derived from the liver during processing and/or storage. This is the first report that the liver of *L. inermis* in Japan is strongly toxic with levels exceeding 1,000 MU/g, to our knowledge. It has been reported that liver of *L. inermis* in Japan is no more than moderately toxic (100–999 MU/g)[2),11)].

Hence, we re-examined the toxicity of *L. inermis* captured off the Japanese coast. The toxicity of 13 specimens is summarized in Table 1. None of the muscle and skin samples tested showed toxicity (<5 MU/g), supporting the view that these tissues can be consumed safely. In contrast, liver, intestine, kidney, gallbladder, spleen and ovary were toxic, although there was a marked individual variation in toxicity. For liver, the highest toxicity score was 199 MU/g. From the standpoint of food safety,

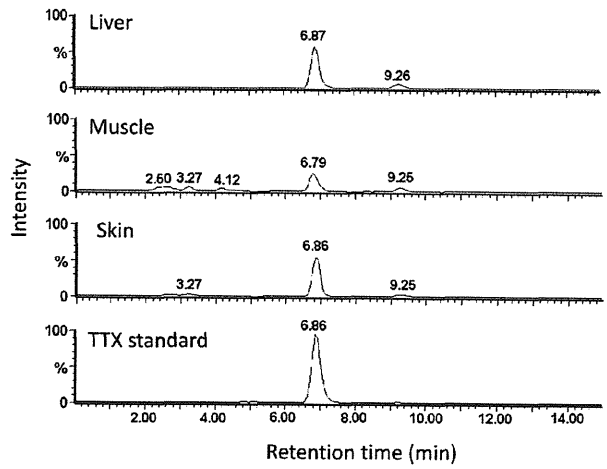


Fig. 3. LC-ESI-MS of the extracts from liver, muscle and skin of the puffer fish sample responsible for the food poisoning

Ten-μL aliquots of dilutions of tissue extracts were subjected to LC-ESI-MS. TTX standard (10 μL) contained 4.4 ng TTX. TTX was monitored in the selected ion recording mode at *m/z* 320, corresponding to the protonated molecular ion $(M+H)^+$.

Table 1. Toxicity of smooth-backed blowfish *Lagocephalus inermis* collected off the Miyazaki coast, Japan

Sample No.	Date of catch	Toxicity (mouse unit/g)								
		Muscle	Skin	Liver	Intestine	Kidney	Gallbladder	Spleen	Ovary	Testis
1	Feb. 2001	<5	<5	199	43.6	21.4	10.2	—	8.0	—*
2	Oct. 2001	<5	<5	11.8	7.0	—	14.4	10.0	—	—
3	Oct. 2008	<5	<5	49.5	8.3	—	—	—	7.8	—
4	Oct. 2008	<5	<5	40.3	8.6	29.1	—	12.3	—	<5
5	Oct. 2008	<5	<5	16.5	<5	59.5	—	17.2	10.0	—
6	Oct. 2008	<5	<5	9.7	<5	9.6	—	5.7	—	<5
7	Oct. 2008	<5	<5	<5	<5	<5	—	—	—	—
8	Nov. 2010	<5	<5	185	14.1	17.7	—	—	—	—
9	Nov. 2010	<5	<5	107	8.7	32.7	—	—	—	—
10	Nov. 2010	<5	<5	58.7	20.9	25.6	—	—	—	—
11	Nov. 2010	<5	<5	58.6	<5	15.0	—	—	—	—
12	Nov. 2010	<5	<5	34.1	<5	14.8	—	—	—	—
13	Nov. 2010	<5	<5	24.6	<5	15.4	—	—	—	—
Highest score				199	43.6	59.5	14.4	17.2	10.0	
Toxic specimens/ test specimens		0/13	0/13	11/13	3/13	9/11	2/2	3/4	1/3	0/2
Toxicity level										
This study		Non	Non	Moderate	Weak	Weak	Weak	Weak	Weak	Non
Tani (1945)		Non	Non	Moderate	Non	Unknown	Unknown	Unknown	Non	Non

*—: Not determined.

toxicity levels of puffer fish are classified as follows: strongly toxic (≧1,000 MU/g tissue), moderately toxic (100–999 MU/g tissue), weakly toxic (10–99 MU/g tissue) and non-toxic (<10 MU/g tissue). Accordingly, three of 13 liver samples were moderately toxic, eight were weakly toxic and only two were non-toxic. Because the intestine and ovary of *L. inermis* in Japan have so far been considered non-toxic [2), 11)], it is important that these organs were determined to be toxic with a maximum toxicity of 43.6 MU/g and 10.0 MU/g, respectively. Although the toxicity of kidney, gallbladder and spleen has not been reported, these tissues were found to be toxic at a relatively high ratio: 100% (2 toxic/2 test) for gallbladder, 82% (9 toxic/ 11 test) for kidney and 75% (3 toxic/4 test) for spleen.

In conclusion, the food poisoning incident in Nagasaki Prefecture, Japan, in 2008, was due to TTX and was caused by ingestion of the toxic liver of *L. inermis*. Consumption of a piece of the causative liver sample (just 8 g) could have been fatal, being equivalent to the estimated minimum lethal dose (10,000 MU) of TTX in human[1)]. In addition, re-examination of the toxicity of *L. inermis* clearly demonstrated that intestine and ovary, previously regarded as non-toxic tissues, were toxic and that kidney, gallbladder and spleen, whose toxicity has been unknown, were also toxic, even though the number of samples was limited. The results in this study suggest that the toxicity of smooth-backed blowfish *L. inermis* in Japan coast has increased since the report by Tani[11)]. Further study is necessary to re-examine the toxicity of puffer fish, including *L. inermis*, using larger numbers of specimens from various sampling locations in order to prevent puffer fish poisoning incidents.

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Screening for toxicity and resistance to paralytic shellfish toxin of shore crabs inhabiting at Leizhou peninsula, China

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ABSTRACT

The situation of the environment contaminated by paralytic shellfish toxin (PST) in Leizhou peninsula, China, has attracted more attention since seafood poisoning occurred occasionally. In this study, we examined the toxicities of shore crab *Leptodius exaratus*, *Thalamita crenata* and *Metopograpsus latifrons* by mouse assay, resistance to PST by lethal test injection with PST, and discussed the toxicity neutralization of their hemolymph. The results showed 12% of shore crabs possessed toxicity of 4.3–4.4 MU/g. The 100% lethal dose of PST for *M. latifrons* was about 2 times of those for the other two crab species. The hemolymphs of the crabs were all able to neutralize PST and tetrodotoxin (TTX) toxicity in different extent. The above results indicate shore crabs at this area are exposed to an environment potentially contaminated with PST and/or TTX, and the toxicity neutralizing efficacy of their hemolymph directly affects their resistance to the toxins.

1. Introduction

Paralytic shellfish toxin (PST) originated from planktons such as dinoflagellate and cyanobacteria is one group of marine bio-toxin families with highly potent neurotoxicity and can be accumulated widely via food chain not only in shellfish, but also in crustacean such as crabs and lobsters, as well as in fish (Etheridge, 2010; Pearson et al., 2010). The expansion in toxin contamination and increase in intoxication frequencies of marine organisms have been found continuously all over the world, and are considered to be related with changes in marine environments caused by eutrophication and human disturbance and their resulted frequent episode of harmful algal blooms (HABs) (Batoréu et al., 2005; Okolodkov, 2005).

Marine crabs, an important group of crustacean, are popular foods in the world. Unfortunately, crab intoxication occurs occasionally (Gonzales and Alcala, 1977; Alcala et al., 1988; Llewellyn et al., 2002; Noguchi et al., 2011). Hashimoto et al. (1969) screened toxicity of crabs in Ryukyu and Amami Island, Japan, and found three xanthid crabs, *Zosimus aeneus*, *Platypodia granulosa* and *Atergatis floridus*, were extremely toxic. Since then, more toxin-containing crab species were reported in many areas such as Australia (Llewellyn and Endean, 1989; Negri and Llewellyn, 1998), Philippines (Yasumura et al., 1986) and Taiwan (Tsai et al., 1996; Ho et al., 2006), among which, the most toxic crab *Zosimus aeneus* was collected from Ishigaki Island, Japan with recorded toxicity of 16,500 MU/g as PST (Koyama et al., 1983a). The toxin compositions including PST and tetrodotoxin (TTX) varied among individuals, regions, and seasons (Noguchi et al., 1983; Daigo et al., 1985; Noguchi et al., 1986; Ho et al., 2006). Therefore, it is speculated that these toxins are exogenous and accumulated via the ecological food chain (Oikawa et al., 2004; Noguchi et al., 2011).

It is well known that PST and TTX could paralyze the neuron fibers by blocking the Na^+ channels and weakening the muscle strength. Animals poisoned with enough toxins will die because of the paralysis of their respiratory. Nevertheless, some animals could accumulate these toxins (Pearson et al., 2010). Toxin-containing crabs usually possess extremely high tolerance to PST or TTX and need higher toxin concentration to block the action potentials of neuron fibers, compared to the toxin-free crabs (Koyama et al., 1983b; Daigo et al., 1987, 1988). It is, however, interesting to note

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that some species of toxin-free shore crabs also exhibited weak tolerance to the toxins. Shore crabs *Hemigrapsus oregonesis* and *Hemigrapsus nudus* in Canada were found resistance to PST, but not TTX. Only when exposed to PST, a PST-binding protein was simultaneously appeared in the crab body (Barber et al., 1988a; 1988b). Another shore crab *Hanguieus sanguineus* in Japan showed high tolerance to TTX but not to saxitoxin (STX) throughout a year, and a TTX-binding protein was expressed in its hemolymph (Yamamori et al., 1992; Nagashima et al., 2002). Overall, the results of screening resistance of toxin-free crabs to PST and/or TTX in the literatures showed that limited crab species possess relative high resistance to PST and/or TTX, while other species were remarkably sensitive to PST and/or TTX (Hwang et al., 1990; Yamamori et al., 1992; Nagashima et al., 1998). Therefore, it is presumed that functional proteins of crabs are involved in the resistance to toxins.

Leizhou peninsula, China, located at the northern of the South China Sea (SCS), is an important aquaculture area, where HABs appeared three times in 1980, 2003 and 2005, respectively (Qian et al., 2000; Wang et al., 2010). Seafood poisoning with paralytic symptoms occurred occasionally for eating crustacean such as *Tachypleus tridentatus* (Li, 1994), fish of *Periophthalmus cantonensis*, Gastropoda such as *Rapana bezoar*. However, toxin-producing planktons have not been found. The screening of plankton species showed that species of red tide plankton increased from 31 at 1987 to 42 at 2003 at this area, and the dominant species were *Skeletonema costatum*, *Asterionella japonica*, *Thalassiothrix frauenfeldii*, *Thalassionema nitzschioides*, etc. (Lü et al., 1994; Zhang, 2004; Wang et al., 2010). Therefore, we speculate that the competitive growth of the toxin-producing planktons may be suppressed by other nontoxic planktons, consequently the cell density of the toxic planktons is too low for observing. In contrast, *Alexandrium tamarense* was found to be one of notorious toxic species at the Daya Bay, about 300 km away from the east of Leizhou peninsula (Wang et al., 2011).

On the other hand, various species of shore crabs inhabiting at this area, such as *Thalamita*, *Leptodius*, *Metopograpsus*, *Mictyris* and *Uca*, seem to be dynamic, regardless whether or not there are red tide damages. How they protect themselves from the toxin harm and even accumulate the toxins has attracted our attention. We have attempted to assay the toxicity of five species of shore crabs, *Thalamita crenata*, *Leptodius exaratus*, *Metopograpsus latifrons*, *Mictyris brevidactylus*, *Uca arcuata*, and other four unknown species of shore crabs, and found most of them possessed weak toxicity, indicating potential toxic contamination in this area. In order to control the situation of the toxic environment and explain the mechanisms why those crabs are not affected by the toxins, we used relative large shore crabs, *T. crenata*, *L. exaratus* and *M. latifrons* as materials to determine their toxicity, resistance to PST, and PST/TTX toxicity neutralization of their hemolymph, and to discuss preliminarily the relationship between the effects of hemolymph and their resistance to PST/TTX.

2. Materials and methods

2.1. Materials

Shore crabs *T. crenata*, *L. exaratus* and *M. latifrons* were collected at the coast of Leizhou peninsula, China (Fig. 1) from December 2009 to March 2012. They were immediately transported to the laboratory of Aquatic Product Advanced Processing Center of Guangdong Higher Education Institutes, Zhanjiang, China, and used to conduct experiments. PST used in this experiment was partially purified from a xanthid crab *Z. aeneus* caught at Ishigaki Island, Okinawa Prefecture, Japan, and contained both saxitoxins and gonyautoxins (Lin et al., 2011). TTX was purchased from Third

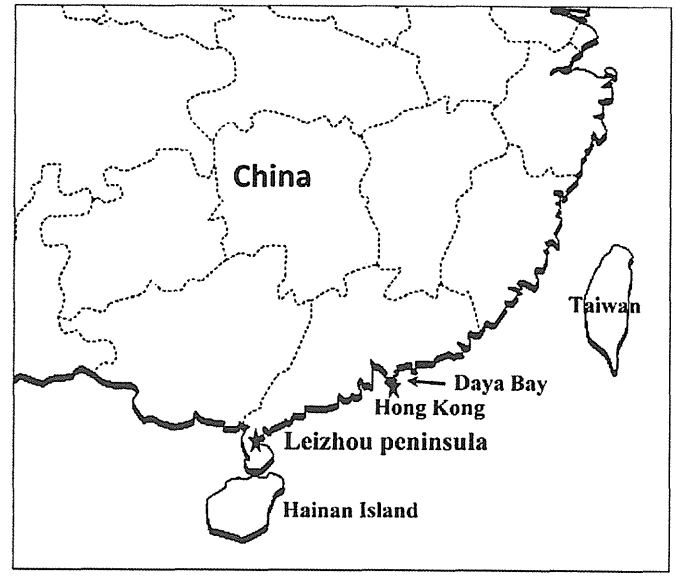

Fig. 1. Location of Leizhou peninsula in China.

Institute of Oceanography, State Oceanic Administration (Xiamen, China). KM strain male mice (20 ± 2 g of body weight) were kindly provided by Shuanglin Biological Medicine Co., Ltd. (Zhanjiang, China). The facilities for experimental animals are fully accredited with a license number of SYXK 2008-0010 and the animal experiments were performed according to the rules and regulations of the Animal Ethics Committee of Guangdong Laboratory Animal Monitoring Institute.

2.2. Toxicity determination

Crab toxicity was individually determined according to AOAC standard method for PST with slight modification (Association of Official Analytical Chemists, AOAC, 2001). Briefly, a crab was ground in a mortar with an equal volume (equivalent to the crab weight) of 0.1 M HCl, and the pH of the mixture was kept approximately to 3.0 by continually adding concentrated HCl during grinding. When the pH of the mixture was stable at around 3.0, the mixture was heated in a boiling water-bath for 5 min. After cooled to room temperature and adjusted to pH 3.0, the mixture was centrifuged at 2200×*g* for 10 min. The supernatant was collected and aliquots of 1 ml were injected intraperitoneally into a group of three KM strain male mice. The mean lethal time was used to calculate the toxicity which is expressed as mouse unit (MU). One MU was defined as the amount of toxin which kills a mouse in 15 min after injection.

2.3. Determination of resistance of crabs to PST

The resistance to PST of crabs *T. crenata* (16.3 ± 6.0 g of body weight), *L. exaratus* (14.1 ± 6.5 g of body weight) and *M. latifrons* (7.2 ± 1.6 g of body weight) was determined according to the method of Daigo et al. (1987). Briefly, PST solution was serially diluted with distilled water. An aliquot of PST solution equivalent to 1% of body weight of the crab was injected at the carpus of the crab's right chela, and the crab was immediately returned to an aquarium. The responses of eye, antenna, chela and leg to physical stimuli were observed to judge whether the crab died, and the death time required was recorded. The dose injected to the crabs was defined as the amount of toxicity (MU) per 20 g of crab (MU/20 g of crab), and the minimum lethal dose (MLD) was used to express the resistance of crabs to PST.

2.4. Preparation of crab hemolymph sample

Crab hemolymph sample was prepared according to the method of Nagashima et al. (2002) with some modifications. Firstly, hemolymph was withdrawn from the chela carpus of the live crab with a syringe, and every 5 ml hemolymph was immediately mixed with 0.2 ml of 0.3 M sodium hydrogen carbonate solution to prevent coagulation. The hemolymph sample was then filtrated with a self-made absorbent cotton filter tube to discard foam and insoluble substance. Approximately 10 ml hemolymph sample was obtained from each crab species for each test.

2.5. Apparent PST and TTX toxicity neutralization of hemolymph sample

Apparent PST and TTX toxicity neutralization of hemolymph sample prepared as above was analyzed by comparing the lethal time of mice in hemolymph test group and control group in mouse bioassay. Briefly, a PST or TTX stock solution was prepared to a suitable concentration, which after diluted three times was able to kill a mouse in 4–6 min. For the hemolymph test group, the PST or TTX test solution was prepared by mixing equal volumes of PST or TTX stock solution, distilled water and hemolymph sample. For the control test, the PST or TTX test solution was prepared by diluting the PST or TTX stock solution three times with distilled water. One ml of PST or TTX test solution was intraperitoneally injected to a mouse, and the lethal time required for each mouse was recorded. Each of the determination was repeated in triplicate. The lethal time of mice in both groups was expressed as mean ± standard deviation and analyzed by *t*-test to examine the significance of differences between the two groups. A p value less than 0.05 was considered as statistical difference.

3. Results

3.1. Toxicities of crabs

Table 1 shows the toxicity of shore crabs *T. crenata*, *L. exaratus* and *M. latifrons*. The toxicities of most individual *T. crenata*, *L. exaratus* or *M. latifrons* crabs in regardless of their size was lower than 4 MU/g, the limiting value for food hygiene, exception one *T. crenata* (specimen No. 4), one *L. exaratus* (specimen No. 11) and one *M. latifrons* (specimen No. 21) with toxicity of 4.4 MU/g, 4.4 MU/g and 4.3 MU/g, respectively. Overall, 12% of shore crabs had higher toxicity than the limiting value for food hygiene. When intraperitoneally inoculated with the extracts from these crabs, mice exhibited neurotoxic signs such as convulsion, jumping and respiratory failure and died. The toxic principles of the crabs were not further explored due to the scarcity of the samples.

3.2. Resisance of shore crabs to PST

Table 2 summarizes the resistance to PST of shore crabs *T. crenata*, *L. exaratus* and *M. latifrons*. The minimum lethal dose (MLD) was (1.5–5.8) MU/20 g for *T. crenata*, (2.8–6.9) MU/20 g for *L. exaratus*, and (2.8–13.0) MU/20 g for *M. latifrons*, respectively.

3.3. Apparent PST toxicity neutralization of hemolymph

Table 3 summarizes the apparent PST toxicity neutralization of hemolymph from shore crabs *T. crenata*, *L. exaratus* and *M. latifrons* and the relative neutralization ratio of toxicity, which was calculated and expressed as percentage ratio of toxicity reduced in the hemolymph test to the corresponding toxicity value in the control test. All the mouse lethal time in the hemolymph test was significantly longer than those in the control test ($p < 0.001$). For *T. crenata*, the mouse lethal time were 3–4 times to that of the control test, except for the datum obtained at 17 December, 2009, which was about 24 times of that of control test. The relative neutralization ratio of toxicity of the hemolymph was ranged from 37.2% to over 49%. For *L. exaratus*, the mouse lethal time in the hemolymph test was approximately 2 times to that of the control test. The relative neutralization ratios of toxicity in the hemolymph test were ranged from 25.9% to 41.1%. For *M. latifrons*, all the tested mice survived, and the relative neutralization ratios of toxicity in the hemolymph test were higher than 52%, the highest in apparent PSP toxicity neutralization among the three crab species.

Table 1
PST toxicities of shore crabs *T. crenata*, *L. exaratus*, *M. latifrons* inhabiting at Leizhou peninsula, China.

Specimen no.	Crab species	Sex[a]	Body weight (g)	Toxicity[b] (MU/g)
1	*T. crenata*	F	3.0	<4.0
2		M	6.8	<4.0
3		F	10.9	<4.0
4		M	14.4	4.4
5		M	14.6	<4.0
6		M	18.0	<4.0
7		F	24.4	<4.0
8		M	38.8	<4.0
9	*L. exaratus*	F	3.7	<4.0
10		M	5.9	<4.0
11		M	10.5	4.4
12		M	13.3	<4.0
13		F	13.4	<4.0
14		M	15.3	<4.0
15		M	15.4	<4.0
16		M	16.8	<4.0
17		F	16.8	<4.0
18		M	63.1	<4.0
19	*M. latifrons*	M	3.8	<4.0
20		M	4.2	<4.0
21		M	4.6	4.3
22		F	4.6	<4.0
23		F	5.1	<4.0
24		M	6.6	<4.0
25		M	6.8	<4.0

[a] M = male, F = female.
[b] The toxicity of individual crabs was examined according to AOAC method for PST and expressed as mouse unite (MU) per gram of the crab, one MU was defined as the amount of toxin which kills a mouse in 15 min after injection.

Table 2
Resistance to PST of shore crabs *T. crenata*, *L. exaratus* and *M. latifrons* inhabiting at Leizhou peninsula, China.

Crab species	Dose[a] (MU/20 g of crab)	No. of dead/No. of examined[b]
T. crenata	1.0	0/4
	1.5	3/4
	2.8	7/9
	5.8	3/3
L. exaratus	1.5	0/17
	2.8	4/14
	5.8	5/11
	6.9	4/4
M. latifrons	1.5	0/7
	2.8	1/7
	5.8	1/8
	12.0	2/4
	13.0	2/3

[a] Dose of PST injected to the crabs was defined as the amount of toxicity (MU) per 20 g of crab (MU/20 g of crab).
[b] The previous data are the number of crabs dead after injection with PST, and behind data are the total number of crabs examined.

Table 3
PST toxicity neutralization of hemolymph from shore crabs *T. crenata*, *L. exaratus* and *M. latifrons* inhabiting at Leizhou peninsula, China.

Crab species	Date of catch	Death time[a] (min)	Relative neutrolization ratio of toxicity[c] (%)
T. crenata	17 December, 2009	142.9 ± 32.1 (6.0 ± 0.2)*	>49
	30 December, 2009	20.3 ± 1.7 (6.0 ± 0.2)*	43.5
	18 November, 2009	24.6 ± 2.1 (6.0 ± 0.2)*	44.2
	4 January, 2010	19.4 ± 2.1 (6.4 ± 0.4)*	37.2
	13 May, 2010	27.9 ± 2.7 (6.1 ± 0.4)*	42.1
L. exaratus	14 December, 2009	8.7 ± 0.8 (5.6 ± 0.2)*	25.9
	17 December, 2009	12.4 ± 0.5 (6.0 ± 0.2)*	38.1
	30 December, 2009	15.9 ± 2.0 (6.0 ± 0.2)*	41.1
	4 January, 2010	14.3 ± 1.2 (6.4 ± 0.4)*	34.6
	13 May, 2010	13.1 ± 1.6 (6.1 ± 0.4)*	36.2
M. latifrons	30 December, 2009	(−)[b] (6.0 ± 0.2)	>52
	13 May, 2010	(−)[b] (6.1 ± 0.4)	>52

*$p < 0.001$, significantly different from that of the control test shown in the parentheses.
[a] The data in parentheses were from the control test under the same experiment conditions, in which distilled water was used instead of the hemolymph.
[b] (−), survival.
[c] The relative neutralization ratio of toxicity which was calculated according to the AOAC method described in the section 2.2 and expressed as percentage ratio of toxicity reduced in the hemolymph test to the corresponding toxicity value in the control test.

3.4. Apparent TTX toxicity neutralization of hemolymph

Table 4 shows the apparent TTX toxicity neutralization of hemolymph of shore crabs *T. crenata*, *L. exaratus* and *M. latifrons*. The lethal times in hemolymph test for *T. crenata* and *M. latifrons* were significantly longer than those of the control test, exception of that of *L. exaratus*. For the *T. crenata*, the lethal time in the hemolymph test prolonged approximately 1.5 times relative to the control test. For the *M. latifrons*, the lethal time in the hemolymph test was more than 2 times to those of the control test.

4. Discussion

As shown in Table 1, the three shore crab species *T. crenata*, *I. exaratus* and *M. latifrons* were all low in toxicities (~4.4 MU/g) in contrast to the toxin-containing xanthid crabs such as *Z. aeneus*, *P. granulosa*, and *A. floridus* (Noguchi et al., 1983, 2011; Tsai et al., 1996). Therefore, they were considered as toxin-free crabs. Despite of this, there were still 12% of the tested shore crabs had toxicity higher than the limiting value for food hygiene (4 MU/g). This result furthermore confirms our speculation that the environment has been potentially contaminated by toxins. Toxin-producing planktons were considered to be the major toxin-producing source in marine environment and intoxicating marine animals via food chain (Pearson et al., 2010). However, toxin-producing planktons have not been found out at this area (Lü et al., 1994; Zhang, 2004; Wang et al., 2010). Although what is the toxin producer remained a mystery, it is necessary to warn the local residents to pay attention to the risk of food intoxication by ingesting those crabs.

For checking whether or not those shore crabs are able to resist to PST, experiments of lethal test injection with PST to crabs were performed. The MLD of *T. crenata*, *L. exaratus* and *M. latifrons* were (1.5–5.8), (2.8–6.9) and (2.8–13.0) MU/20 g crabs, respectively (Table 2). These values were relative higher than other sensitive crab species (Hwang et al., 1990; Yamamori et al., 1992; Nagashima et al., 1998). Particularly, the LD_{100} of *M. latifrons* (>13.0 MU/20 g of crab) was about 2 times of those of the other two crab species, nevertheless their toxicities were similar. This result indicates the three shore crabs are all resistant to PST with different extent. Koyama et al. (1983b) compared resistance to PST between toxin-containing crabs and toxin-free crabs, and found all of the toxin-free shore crabs were sensitive to PST with an MLD value less than 1 MU/20 g of crab, while the toxin-containing crabs were extremely resistant to PST with an MLD value of (5000–10,000) MU/20 g of crab. The high resistance to PST of the toxin-containing crabs was attributed to the insensitivity of nerve cells to the toxin (Daigo et al., 1988). On the other hand, toxin-free crabs *H. oregonesis* and *H. nudus* became resistant to the PST while exposed to PST (Barber et al., 1988a), and a novel high molecular protein appeared (Barber et al., 1988b). It seems there is another function involved in the resistance to toxins for the toxin-free crabs.

To confirm whether or not there is a relationship between the effects of hemolymph and resistance to PST, the apparent PST toxicity neutralization of hemolymph was analyzed. All the mouse lethal time in the hemolymph test was significantly longer than that of the control test ($p < 0.001$), indicating that hemolymph plays an important role in toxicity neutralization. Consistent with the above results, *M. latifrons* showed the highest apparent toxicity neutralization ability among the three crab species, because all the tested mice treated with its hemolymph survived. These results implicate that the toxicity neutralizing efficacy of hemolymph directly affects the resistance of crabs to PST. Since some crabs were reported to have TTX as well as PST (Noguchi et al., 1986; Yasumura et al., 1986), the apparent TTX toxicity neutralization of hemolymph has been analyzed. The mouse lethal time in the hemolymph test for *T. crenata* and *M. latifrons* was significantly longer than that of the control test ($p < 0.05$). This result indicates that hemolymph of *T. crenata* and *M. latifrons* are also able to neutralize TTX.

The above results clarify that the resistance to PST of shore crabs closely depends on the toxicity neutralization of their hemolymph. The functional compositions neutralizing toxicity will be further investigated in detail.

Table 4
TTX toxicity neutralization of hemolymph from shore crabs *T. crenata*, *L. exaratus* and *M. latifrons* inhabiting at Leizhou peninsula, China.

Crab species	Date of catch	Death time[a] (min)
T. crenata	28 June, 2011	5.4 ± 0.2 (3.6 ± 0.3)*
L. exaratus	19 March, 2012	5.9 ± 1.3 (4.6 ± 0.3)
M. latifrons	17 January, 2012	8.6 ± 1.2 (3.6 ± 0.3)*

*$p < 0.05$, significantly different from that of the control test shown in the parentheses.
[a] The data in parentheses were from the control test under the same experiment conditions, in which distilled water was used instead of the hemolymph.

5. Conclusion

Shore crabs *T. crenata*, *L. exaratus* and *M. latifrons* are considered as toxin-free crabs even though 12% of them possess toxicity slightly higher than the limiting toxicity of 4 MU/g for food hygiene. All the three shore crabs are resistant to PST. In particular, the hemolymph of crab *M. latifrons* plays an important role in PST and TTX toxicity neutralization. These results indicate that shore crabs at the Leizhou peninsula were exposed to an environment potentially contaminated with PST and/or TTX, and the toxicity neutralizing efficacy of their hemolymph directly affects their resistance to PST and/or TTX.