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Histamine level and species identification of billfish meats implicated in two food-borne poisonings

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Abstract

Two incidents of food-borne poisonings, causing illness in 59 and 43 victims due to ingestion of billfish meats, occurred in May 2004, in Pingtung, southern Taiwan and in December 2004, Taichung, central Taiwan, respectively. One fried billfish fillet and five frozen billfish fillet samples collected, respectively, from the suspected restaurants in Pingtung and Taichung, respectively, were tested to determine the histamine levels and identify fish species. Analyses of histamine showed that the suspected billfish samples in two food poisonings contained more than 150 mg/100 g of histamine, which is higher than the hazard action level of 50 mg/100 g. Judging from the allergy-like symptoms of the victims and the high histamine levels in the suspected billfish samples, both food-borne poisonings were strongly suspected to be caused by histamine intoxication. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to identify the species of the suspected billfish samples in both food poisonings. The 348 bp amplified fragment of the mitochondrial cytochrome *b* gene by PCR was digested with *Bsa*JI, *Cac*8I and *Hpa*II enzymes to distinguish the species of the suspected billfish samples. Consequently, the species of Pingtung and Taichung billfish samples implicated in food poisonings were identified as *Makaira nigricans* and *Xiphias gladius*, respectively.

Keywords: Histamine; Food poisoning; Billfish; Polymerase chain reaction; Cytochrome *b* gene

1. Introduction

Histamine is the causative agent of scombroid poisoning, a food-borne chemical hazard. Scombroid poisoning is usually a mild illness with a variety of symptoms, including rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin (Taylor, 1986). Severity of the symptoms can vary considerably with the amount of histamine ingested and the individual's sensitivity to histamine (Russsell & Maretic, 1986). Scombroid fish, such as tuna, mackerel, bonito and saury, that contain high levels of free histidine in their muscle are often implicated in scombroid poisoning incidents (Taylor, 1986). However, several species of non-scombroid fish, such as mahi-mahi, bluefish, herring, and sardine have often been implicated in incidents of scombroid poisoning (Price & Melvin, 1994). In Taiwan, scombroid poisoning occurs occasionally (Chen & Malison, 1987; Murray, Hobbs, & Gilbert, 1982; Tsai et al., 2005), and the fish implicated in these outbreaks are tuna, mackerel, and black marlin. Recently, due to their popularity among Taiwanese people, sailfish and marlin fillets have become the most frequently implicated fish species in scombroid outbreaks in Taiwan (Hwang, Chang, Shiau, & Chai, 1997; Hwang, Chang, Shiau, & Cheng, 1995; Hwang et al., 1999).

However, the fish species implicated in these food poisoning incidents were not identified because the causative

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leftovers had been processed and cooked. Recently, it has been shown that analysis of mitochondrial (mt) DNA successfully differentiated species of fish (Bartlett & Davidson, 1991; Desall & Birstein, 1996; Mackie et al., 1999). Polymerase chain reaction (PCR) amplification and restriction enzyme analysis of the cytochrome *b* gene have also been used for identification of fish species (Cespedes et al., 1998; Ram, Ram, & Baidoun, 1996). Recently, we demonstrated that five billfish species, including *Xiphias gladius* (swordfish), *Makaira nigricans* (blue marlin), *Makaira indica* (black marlin), *Istiophorus platypterus* (sailfish) and *Tetrapturus audax* (striped marlin), could be identified by using PCR amplification and restriction enzyme analysis of the cytochrome *b* gene (Hsieh, Chai, & Hwang, 2005, 2007).

Two incidents of food-borne poisonings, causing illness in 59 and 43 victims due to ingestion of billfish meats, occurred in May 2004, in Pingtung, southern Taiwan and in December 2004, Taichung, central Taiwan, respectively. Victims all suffered from allergy-like symptoms, including rash, nausea, diarrhea, and flushing, but all recovered within 24 h. To elucidate the causative agent, the suspected billfish samples collected from the suspected restaurants were analyzed for histamine levels. In addition, PCR amplification of mt DNA sequence and restriction enzyme analysis were used to identify the species of the suspected billfish samples.

2. Materials and methods

2.1. Preparation of samples

One fried billfish fillet and five frozen billfish fillets were collected, respectively, from the suspected restaurants in Pingtung and Taichung County where the victims ate the suspected billfish fillets that caused the poisoning. After collection, all suspected samples were stored at −20 °C prior to use.

2.2. Biogenic amines analysis

Each suspected billfish sample was ground in a Waring Blender for 3 min. The ground samples (5 g) were transferred to 50 ml centrifuge tubes and homogenized with 20 ml of 6% trichloroacetic acid (TCA) for 3 min. The homogenates were centrifuged (10,000*g*, 10 min, 4 °C) and filtered through Whatman No. 2 filter paper (Whatman, Maidstone, England). The filtrates were then placed in volumetric flasks, and TCA was added to bring to a final volume of 50 ml. Samples of standard biogenic amine solutions and 2 ml aliquots of the fish sample extracts were derivatized with benzoyl chloride according to the previously described method (Hwang et al., 1997). The benzoyl derivatives were dissolved in 1 ml of methanol, and 20 μl aliquots were used for HPLC injection.

The contents of biogenic amines in the suspected billfish samples were determined with a Hitachi liquid chromatograph (Hitachi, Tokyo, Japan), consisting of a Model L-7100 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–Vis detector (set at 254 nm), and a Model D-2500 Chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 × 4.6 mm, E. Merck, Damstadt, Germany) was used for chromatographic separation. The gradient elution programme began with 50:50 (v/v) methanol:water at a flow rate of 0.8 ml/min for the first 0.5 min, followed by a linear increase to 85:15 methanol:water (0.8 ml/min) during the next 6.5 min. The methanol:water mix was held constant at 85:15 (0.8 ml/min) for 5 min, and then decreased to 50:50 (0.8 ml/min) during the next 2 min.

2.3. DNA extraction and PCR amplification

DNA was extracted according to the protocol described in our previous study (Hsieh et al., 2005, Hsieh, Chai, & Hwang, 2007). In brief, about 0.3 g of sample was homogenized with the extraction buffer (50 mM Tris–HCl, pH 8.0, 0.1 M EDTA, 1% SDS, and 0.2 M NaCl) containing 50 μl of 5 mg/ml proteinase K (Amresco, Solon, Ohio, USA). The samples were incubated overnight at 55 °C with shaking. After incubation, tubes were placed on ice for 30 min, centrifuged at 12,000*g* for 10 min and supernatant was transferred to a clean tube. DNA was extracted once with phenol, twice with phenol–chloroform–isoamyl alcohol in a 25:24:1 ratio and once with chloroform, and then precipitated twice with ethanol at −20 °C. The dried pellets were resuspended in 50–100 μl of sterilized distilled water and concentration of extracted DNA was estimated by absorbance at 260 nm.

The PCR primers L-CYTBF 5′-GCT ATR CAC TAY ACM TCR GAC-3′ and H-CYTBF 5′-GCC TCC TCA RAT TCA TTG GAC-3′ (R: A or G; Y: C or T; M: A or C) specific for cyt *b* gene of fish were designed in this laboratory and used to amplify a 348 bp fragment in PCR (Hsieh et al., 2005, 2007). The PCR amplification reactions were performed in a total volume of 100 μl. Each reaction mixture contained 100 ng of extracted template DNA, 0.2 μM of each primer, 200 μM of each dNTP and 2.5 U of Pro Taq DNA polymerase (Amresco, Solon, Ohio, USA) in a reaction buffer containing 20 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1% Triton X-100 and 0.1 mM dithiothreitol (DTT). PCR reaction was carried out in a Gene-Amp PCR system 2400 (Perkin Elmer, Foster City, CA) programmed to perform a denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of 1 min at 95 °C, 1 min at 50 °C and 2 min at 72 °C. The last extension step was extended to 10 min longer.

2.4. DNA purification and restriction site analysis of PCR products

PCR product (6 μl) was loaded onto a 2% agarose gel containing 1 μg/ml of ethidium bromide in TBE buffer and electrophoresed at 50 V for 120 min. The DNA band

was excised under UV light and melted in 5 volumes of Tris EDTA (TE) buffer at 65 °C for 5 min. DNA was extracted twice with phenol, once with 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. The dried pellet was resuspended in 20 μl of sterilized distilled water. The concentration and quality of the DNA were estimated by agarose gel electrophoresis using a 2 μl of sample. Purified PCR products were sequenced at Mission Biotech (Taipei, Taiwan), using the above primers and the ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer/Applied Biosystems Division, Foster City, CA) in an ABI PRISM 377-96 DNA sequencer (Perkin–Elmer/Applied Biosystems Division, Foster City, CA). Two replicate sequences were obtained from the Wisconsin Package, Version 10 (Genetics Computer Group, 2000, San Diego, CA). The amplified PCR products were analyzed by agarose gel electrophoresis (Hsieh et al., 2005, 2007).

As described previously (Hsieh et al., 2005, 2007), the endonuclease *Bsa*JI, *Cac*8I and *Hap*II (Promega, Madison, WI., USA) were tested for restriction analysis of the amplified PCR products. Digests were performed in a 10 μl volume with 100–200 ng of the amplified DNA, 5 U of enzyme, and 1:10 diluted manufacturer's 10X digestion buffer, and bovine serum albumin (BSA). Digestion mixtures were incubated for 2 h at 60 °C (*Bsa*JI) or at 37 °C (*Cac*8I and *Hpa*II). The resulting fragments were separated by electrophoresis in a 2.0% agarose gel containing 10 μg/ml of ethidium bromide for 1 h at 100 V. The sizes of the resulting DNA fragments were estimated by comparing with those of a commercial 100-bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

3. Results and discussion

Levels of biogenic amines are summarized in Table 1. Of the nine biogenic amines checked, only low levels of cadaverine and high levels of histamine were detected in all tested billfish samples. The suspected billfish sample from Pingtung had a histamine level of 257 mg/100 g, while five suspected billfish samples from Taichung had 157–270 mg/100 g of histamine. The US Food & Drug Administration (FDA) has established a "hazard action level" of 50 mg histamine/100 g (500 ppm) for fish, based on data collected from numerous outbreaks (Taylor, 1989). Bartholomev, Berry, Rodhouse, and Gilhouse (1987) demonstrated that histamine at greater than 100 mg/100 g in fish would be toxic and unsafe for consumption. Thus, high levels of histamine in the suspected billfish samples, along with the allergy-like symptoms developed in the victims, supported the conclusion that histamine was the causative agent of the both food-borne poisoning incidents.

High levels of histamine have been found in various types of fish implicated in scombroid poisoning. The marlin *M. indica*, implicated in a poisoning incident, had a histamine level ranging between 93.5 and 276 mg/100 g (Morrow, Margolies, Rowland, & Robert, 1991); the hot-smoked mackerel, *Scomber scombrus*, implicated in a scombrotoxic incident, had a histamine level of 270 mg/100 g (Clifford, Walker, & Wright, 1989); the canned tuna implicated in a poisoning had a histamine level of 116 mg/100 g, while that of wholesome canned tuna had only 2.74 mg/100 g (Kim & Bjeldanes, 1979). In Taiwan, incidences of scombroid poisoning have only occurred occasionally, and the fish implicated in those occasional outbreaks are tuna, mackerel, and black marlin (Chen & Malison, 1987; Murray et al., 1982; Tsai et al., 2005). Sailfish, *I. platypterus*, and marlin fillets have recently become the most frequently implicated fish species in scombroid outbreaks in Taiwan (Hwang et al., 1995, 1997, 1999). Quality loss and histamine accumulation often occur after frozen fish of the above-mentioned species are thawed and kept for long periods of time at room temperature before further processing. Since histamine is heat-resistant, it can remain intact in canned or cooked fish products (Lopez-Sabater, Rodriguez-Jerez, Hernandez-Herrero, & Mora-Ventura, 1994). In this study, the use of poor quality fish as raw material for cooking results in the presence of toxic levels of histamine in the billfish fillets.

The DNA extracts from the suspected billfish samples were tested for PCR amplification with the L-CYTBF

Table 1
The levels of biogenic amines in the suspected billfish samples collected from Pingtung and Taichung, implicated in two food poisonings

Source	Levels of biogenic amines (mg/100 g)								
	Put[a]	Cad	Tpm	Phe	Spm	Spm	Hm	Tym	Agm
Pingtung, May 2004									
P-1	ND [b]	0.8	11.6	37.3	24.3	ND	257.3	ND	ND
Taichung, December 2004									
T-1	ND	6.6	7.8	ND	ND	ND	202.5	0.3	ND
T-2	ND	11.9	ND	ND	ND	ND	193.1	ND	ND
T-3	1.6	5.7	ND	ND	ND	4.9	269.5	4.3	ND
T-4	ND	8.0	1.4	ND	ND	ND	189.2	ND	ND
T-5	2.1	4.8	ND	ND	ND	ND	157.0	ND	ND

[a] Put: putrescine; Cad: cadaverine; Tpm: tryptamine; Phe: 2-phenylethylamine; Spd: spermidine; Spm: spermine; Hm: histamine; Tym: tyramine; and Agm: agmatine.
[b] ND: Not detected (amine level less than 0.1 mg/100 g).

and H-CYTBF primers. Subsequently, the electrophoretic analysis of the PCR products from suspected billfish samples all had a 348 bp fragment (Fig. 1). According to our previous study (Hsieh et al., 2005, 2007) the restriction enzymes, *Bsa*JI, *Cac*8I and *Hap*II, were found to be useful because of their convenience and speed in identifying five billfish meat species. The endonuclease,*Bsa*JI, separated four billfish species into two groups, *M. nigricans*/*M. indica* (348 bp) and *X. gladius*/*I. platypterus* (204 and 144 bp). However, the PCR products of *T. audax* meats, digested with restriction enzyme *Bsa*JI, produced two fragments (334 and 14 bp). The 14 bp DNA fragment was difficult to visualize on 2% agarose gel, thereupon *T. audax* was classified as the non-digested group (*M. nigricans*/*M. indica*) (Fig. 2). Moreover, the five billfish species could be separated into "digested" and "non-digested" by restriction enzyme *Cac*8I. The PCR product of *X. gladius* was digested for 253 and 95 bp by *Cac*8I, but that of *I. platypterus* was not digested by *Cac*8I. Similarly, the PCR product of *M. indica* was digested for 253 and 95 bp by *Cac*8I, but that of *M. nigricans* and *T. audax* was not digested by *Cac*8I (Fig. 2). The PCR products of *M. nigricans* and *T. audax* species could not be distinguished by restriction enzymes, *Bsa*JI and *Cac*8I, so another restriction enzyme, *Hpa*II, was used to separate *M. nigricans* and *T. audax*. The PCR products from *T. audax* meats produced two fragments (291 and 57 bp) and *M. nigricans* was not digested by *Hap*II (Fig. 2). Therefore, fish species identification of the six suspected billfish samples implicated in both food-borne poisonings could be distinguished by RFLP patterns generated by amplifying the 348 bp region of the mt cyt *b* gene and subsequent restriction enzyme digestion using the restriction enzymes, *Bsa*JI, *Cac*8I and *Hpa*II. It was found that there was no restriction site for *Bsa*JI, *Cac*8I and *Hpa*II in the PCR product of the suspected billfish sample from Pingtung (lane P-1) (Figs. 3–5). However, a single restriction site for *Bsa*JI and *Cac*8I was found in PCR products of the suspected billfish samples from Taichung (lane T-1–5) (Figs. 3 and 4). Judging from the PCR-RFLP pattern, the fish species of the sample from Pingtung was identified as *M. nigricans*, whereas the fish species of the samples from Taichung was identified as *X. gladius*.

Because the suspected fish samples implicated in histamine poisoning are usually heavily heated, the proteins in the fish samples have degraded and almost all proteins were

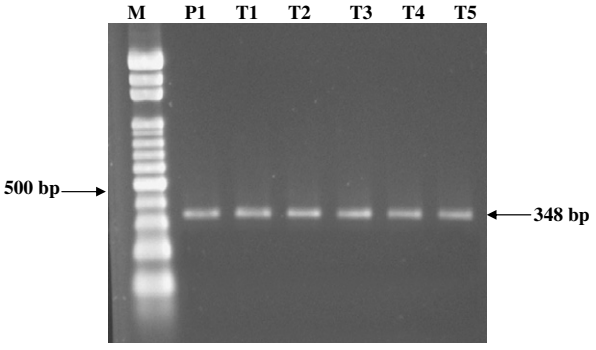

Fig. 1. Electrophoretic analysis of PCR products of the 348 bp on cytochrome *b* gene on 2.0% agarose gel. *M* = molecular weight markers, Bio 100-bp DNA Ladder. Lanes: P-1 = suspected billfish sample from Pingtung, T-1–5 = suspected billfish samples from Taichung.

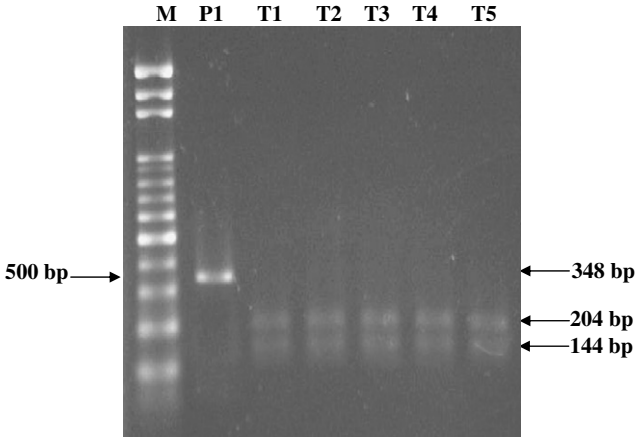

Fig. 3. Electrophoretic analysis of PCR products of the 348 bp on cytochrome *b* gene digested with *Bsa*JI on 2.0% agarose gel. *M* = molecular weight markers, Bio 100-bp DNA Ladder. Lanes: P-1 = suspected billfish sample from Pingtung, T-1–5 = suspected billfish samples from Taichung.

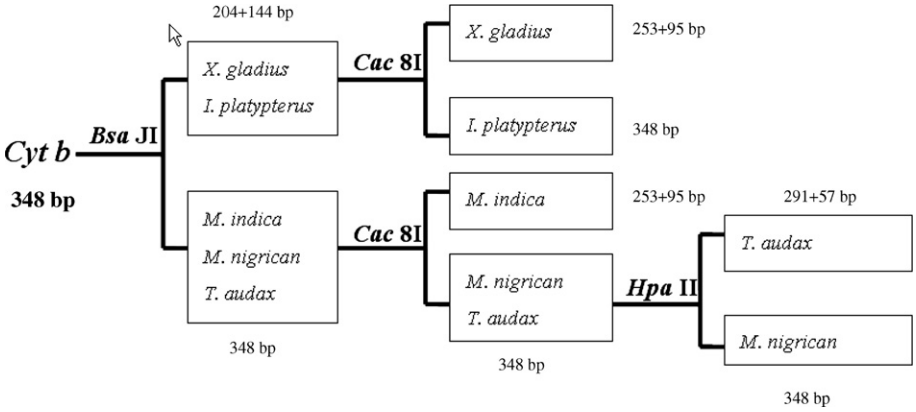

Fig. 2. Key to distinguish species of billfish based on the PCR product of 348 bp on the mt cyt *b* gene.

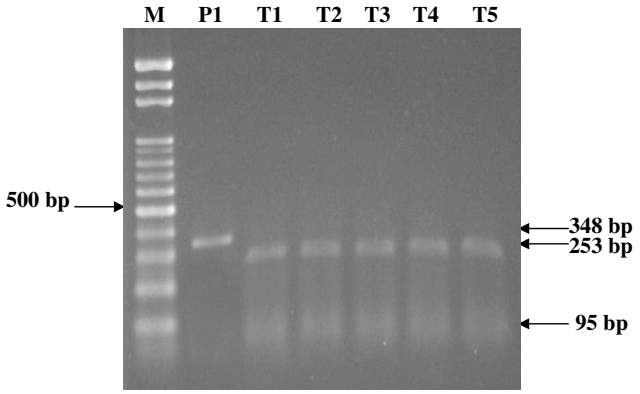

Fig. 4. Electrophoretic analysis of PCR products of the 348 bp on cytochrome *b* gene digested with *Cac*8I on 2.0% agarose gel. *M* = molecular weight markers, Bio 100-bp DNA Ladder. Lanes: P-1 = suspected billfish sample from Pingtung, T-1–5 = suspected billfish samples from Taichung.

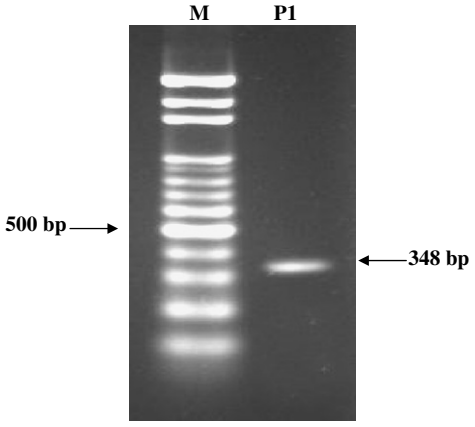

Fig. 5. Electrophoretic analysis of PCR products of the 348 bp on cytochrome *b* gene digested with *Hpa*II on 2.0% agarose gel. *M* = molecular weight markers. Bio 100-bp DNA Ladder. Lane: P-1 = suspected billfish sample from Pingtung.

denatured and damaged. Therefore, protein analysis methods for fish species identification, such as SDS-PAGE, IEF and 2-DE are inappropriate. It is by the most common DNA-based technique used for fish species identification that scientists draw great attention to the mt cyt *b* gene. The species-specific gene appears to have substantial inter- and intra-species variation in its original nucleotide sequence, and the level of variation within species is much less than that between species (Mackie et al., 1999). In this study, we have used the PCR-RFLP method to identify the species of suspected fish. As the result shows, the method is an easy, rapid, cheap and sensitive technique to clearly detect the species of billfish samples implicated in histamine poisoning.

4. Conclusion

This study showed that the suspected billfish samples from Pingtung and Taichung, implicated in both food poisonings, had more than 150 mg/100 g of histamine; hence the high level of histamine in suspected samples could be the etiological factor for both fish-borne poisonings. The method of PCR-RFLP was used to distinguish the species of suspected billfish samples. The suspected billfish species of Pingtung's food poisoning incident was identified as *M. nigricans*, whereas that of Taichung's food poisoning incident was identified as *X. gladius*.

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