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Original Article

Determination of histamine in milkfish stick implicated in food-borne poisoning

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ABSTRACT

An incident of food-borne poisoning causing illness in 37 victims due to ingestion of fried fish sticks occurred in September 2014, in Tainan city, southern Taiwan. Leftovers of the victims' fried fish sticks and 16 other raw fish stick samples from retail stores were collected and tested to determine the occurrence of histamine and histamine-forming bacteria. Two suspected fried fish samples contained 86.6 mg/100 g and 235.0 mg/100 g histamine; levels that are greater than the potential hazard action level (50 mg/100 g) in most illness cases. Given the allergy-like symptoms of the victims and the high histamine content in the suspected fried fish samples, this food-borne poisoning was strongly suspected to be caused by histamine intoxication. Moreover, the fish species of suspected samples was identified as milkfish (*Chanos chanos*), using polymerase chain reaction direct sequence analysis. In addition, four of the 16 commercial raw milkfish stick samples (25%) had histamine levels greater than the US Food & Drug Administration guideline of 5.0 mg/100 g for scombroid fish and/or products. Ten histamine-producing bacterial strains, capable of producing 373–1261 ppm of histamine in trypticase soy broth supplemented with 1.0% L-histidine, were identified as *Enterobacter aerogenes* (4 strains), *Enterobacter cloacae* (1 strain), *Morganella morganii* (2 strains), *Serratia marcescens* (1 strain), *Hafnia alvei* (1 strain), and *Raoultella orithinolytica* (1 strain), by 16S ribosomal DNA sequencing with polymerase chain reaction amplification.

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1. Introduction

Histamine is the causative agent of scombroid poisoning, a food-borne chemical hazard that usually causes a mild illness with a variety of symptoms including rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin [1]. Severity of the symptoms can vary considerably with the amount of histamine ingested and individuals' sensitivity to histamine. Scombroid fish such as tuna, mackerel, bonito, and saury, which contain high levels of free histidine in their muscle tissue, are often implicated in scombroid poisoning incidents [1]. However, several species of nonscombroid fish, such as mahi-mahi, bluefish, herring, and sardine, have also often been implicated in incidents of scombroid poisoning. In Taiwan, scombroid poisoning has occurred occasionally [2–4], and the fish implicated in these outbreaks were tuna, mackerel, milkfish, and black marlin. Recently, due to their popularity with Taiwanese consumers, swordfish and marlin fillets have become the most frequently implicated fish species in scombroid outbreaks in Taiwan [5–7].

Biogenic amines are formed mainly through decarboxylation of specific free amino acids by exogenous decarboxylases released by the microbial species associated with seafood. Many bacterial species are known to possess histidine decarboxylase and have the ability to produce histamine [8]. In addition to *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei*, which have been isolated from the fish incriminated in scombroid poisoning [9], several species of the enteric bacteria capable of producing histamine have also been isolated from fish [10,11]. These include *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *Serratia liquefaciens*, and *Citrobacter freundii* [12,13]. Other than the enteric bacteria, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lowffi*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Aeromonas* spp., and *Photobacterium* spp. have also been reported as histamine producers [10,14]. Our research group also isolated several prolific histamine-forming bacteria, including *Enterobacter*, *Klebsiella*, *Raoultella*, and *Citrobacter* spp. from sailfish fillets, dried milkfish, tuna dumpling, and tuna sandwich in Taiwan [2,15–18].

Milkfish (*Chanos chanos*) is an important aquacultured fish in the Indo-Pacific region, particularly the Philippines, Indonesia, and Taiwan [19]. Histidine at approximately 441 mg/100 g is the most prominent free amino acid found in the white muscle of milkfish, accounting for 80% of the total free amino acids in the fish [20]. Tsai et al [21] reported that milkfish was a better substrate than sailfish for histamine formation by bacterial histidine decarboxylation at elevated temperatures (>15°C). Our research group first reported that dried milkfish products could cause histamine intoxication (the food-borne poisoning incident occurred in February 2006, in southern Taiwan), and *Raoultella ornithinolytica* was the major histamine-producing bacterium responsible for the high content of histamine in the implicated milkfish sample [4].

An incident of food-borne poisoning due to ingestion of fried fish sticks occurred in Tainan city, southern Taiwan, in September 2014. The incident caused 37 victims to fall ill. They all experienced allergy-like symptoms, including rash, nausea, diarrhea, and flushing, however, all recovered within 24 hours. To elucidate the causative agent, two suspected fried fish sticks collected from the suspected kitchen and 16 other raw fish stick samples purchased from retail stores in southern Taiwan were analyzed for levels of biogenic amines, aerobic plate count (APC), total coliforms (TC), *Escherichia coli*, total volatile basic nitrogen (TVBN), and histamine-forming bacteria. Polymerase chain reaction (PCR) amplification of mitochondrial DNA sequence analysis was used to identify the species of the suspected fish sample.

2. Materials and methods

2.1. Samples

Two leftover fried fish sticks, which were associated with the September 2014 poisoning incident, were collected from the elementary school kitchen in Tainan City. In addition, 16 raw fish stick samples of the same fish species as that of the leftover fish sticks were obtained from 16 retail stores (including the suspected supplying store) in Tainan city, in order to determine the overall quality of fish stick products in Tainan city. Prior to purchase, the raw fish stick samples were all processed by processing factories, kept at frozen or refrigeration temperature, and brought to retail markets for sale (300 g each sample/bag). All samples were collected in aseptic bags, placed on ice, and immediately transported to the laboratory for analysis.

2.2. Determination of pH value and salt content

The fish samples (10 g) were homogenized in sterile blenders (Omni International, Waterbury, CT, USA) with 10 mL of distilled water to make a thick slurry. The pH of this slurry was then measured using a Corning 145 pH meter (Corning Glass Works, Medfield, MA, USA). The salt content in each sample was determined by homogenizing 2 g of fish sample with 18 mL of distilled water, and then titrated with 0.1M silver nitrate ($AgNO_3$) using 10% w/v potassium chromate (K_2CrO_4) solution as the indicator.

2.3. Microbiological analysis and isolation of histamine-forming bacteria

A 25-g portion of the fish sample was homogenized at a high speed for 2 minutes in a sterile blender with 225 mL of sterile potassium phosphate buffer (0.05M, pH 7.0). The homogenates were serially diluted with a sterile phosphate buffer, and 1.0 mL aliquots of the dilutes were poured onto Petri dishes (9 cm diameter). Then, 15–20 mL of APC agar (Difco; BD, Sparks, MD, USA) containing 0.5% NaCl at 45–50°C was added and gently mixed. The poured plates were allowed to solidify under a biological clean bench. Bacterial colonies were counted after the plates were incubated at 35°C for 48 hours. Bacterial numbers in the tuna dumpling samples were expressed as $\log_{10}$ colony-forming units (CFU)/g [22].

To isolate histamine-forming bacteria, a 0.1-mL aliquot of the sample dilute was spread on histamine-forming

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bacterium isolation agar fortified with L-histidine [23]. Following incubation of the differential agar plates for 4 days at 35°C, colonies with blue or purple color on the plates were picked and further streaked on trypticase soy agar (Difco; BD) to obtain pure cultures. To determine the ability of each isolated culture to produce biogenic amines, the isolates were inoculated in trypticase soy broth (TSB; Difco; BD) supplemented with 1% L-histidine (TSBH), and then incubated without shaking at 35°C for 24 hours. One milliliter of the culture broth was withdrawn for the quantitation of biogenic amines.

Analyses of TC and *E. coli* in fish samples were conducted using the three-tube most probable number (MPN) method [24]. Lauryl sulfate tryptose broth and brilliant green lactose bile (2%) broth (BGLB broth) incubated at 35°C for 48 hours were used for presumptive and confirmation tests for TC, respectively. Then the culture showed growth and gas production in the BGLB broth is transferred into *E. coli* broth and incubated at 44.5 °C for 24–48 hours. Cultures that showed positive production of gas in BGLB and *E. coli* broth were then confirmed for the existence of *E. coli* by eosine methylene blue agar and by indole, methyl red, Voges–Proskauer, and citrate tests [24].

2.4. Identification of histamine-forming isolates

The presumptive histamine-forming isolates were identified on the basis of morphology, Gram stain, endospore stain, catalase, and oxidase reaction. The identity of histamine-forming isolates was further confirmed by amplifying and sequencing approximately 1400 bp of the 16S ribosomal DNA for bacteria [25,26]. Amplification of histamine-forming bacteria was performed using the universal primers UNI-L (5′-AGAGTTTGATCATGGCTCAG-3′) and UNI-R (5′-GTGTGACG-GGCGGTGTGTAC-3′) [25,26]. Bacterial cells were cultured overnight in 2 mL of TSB at 35°C and then centrifuged at 5000*g* for 10 minutes. The cell pellet was washed and resuspended in 0.5 mL of Tris-EDTA buffer (10mM Tris-HCl and 1mM EDTA at pH 8.0), and then lysed by 20% sodium dodecyl sulfate. The solution was boiled for 20 minutes, and the cellular debris was discarded after centrifugation at 13,000*g* for 3 minutes. The total DNA in the supernatant was thereafter precipitated with 70% ethanol and used as the template DNA for PCR.

PCR amplification was performed in 20 μL reaction mixture containing 10mM Tris-HCl (pH 8.3), 50mM potassium chloride (KCl), 1.5mM magnesium chloride ($MgCl_2$), 20 pmol of each primer, 0.4 μL of each of the four deoxynucleotide triphosphates (each of 0.2mM concentration), 0.4 μL of 0.5 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 1.2 μL of the template DNA (10 ng). Amplifications were performed for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds) in a GeneAmp PCR 2400 Thermal Cycler (Applied Biosystems) with initial denaturation at 94°C for 4 minutes and a final extension at 72°C for 7 minutes [25,26]. Amplicons were detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) eluted in Tris-HCl (10mM, pH 8.5) prior to sequencing. The amplified DNA was directly sequenced with the ABI TaqDye Deoxy Terminator Cycle sequencing kit (Applied Biosystems) and the ABI Model 377 automated DNA sequencer (Applied Biosystems). The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) tool (National Center for Biotechnology Information, Bethesda, MD, USA) to identify histamine-forming bacteria.

2.5. Determination of TVBN

The TVBN content of the fish samples was measured by the method of Conway's dish for triplicate determinations [27]. TVBN extract of the fish samples in 6% trichloroacetic acid (Sigma, St Louis, MO, USA) was absorbed by boric acid and then titrated with 0.02N hydrogen chloride (HCl). The TVBN content was expressed in milligrams/100 g fish.

2.6. Biogenic amine analysis

In total, the content of nine biogenic amines (putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermidine, spermine, histamine, tyramine, and agmatine) in the fish samples was determined by triplicate assays. Each fish sample was ground in a Waring Blender (Oster Co., Milwaukee, WI, USA) for 3 minutes. The ground samples (5 g) were transferred to 50-mL centrifuge tubes, 20 mL of 6% trichloroacetic acid was added, and the mixture was homogenized (Omni International) for 3 minutes. The homogenate was centrifuged (10,000*g*, 10 minutes, 4°C) and filtered through a Whatman No. 2 filter paper (Whatman, Maidstone, England). The filtrate was then placed in a volumetric flask, and trichloroacetic acid was added to bring the final volume to 50 mL. Samples of standard biogenic amine solutions and 1-mL aliquots of the fish sample extracts were derivatized with dansyl chloride according to the previously described method [6]. One milliliter of each culture TSBH broth of a presumptive histamine-forming isolate was also dansylated using the same procedure for fish sample extracts. The dansyl derivatives were filtrated through a 0.45-μm filter, and 20-μL aliquots were used for high-performance liquid chromatography injection.

The contents of biogenic amines in the fish samples were determined using a high-performance liquid chromatography system (Hitachi, Tokyo, Japan) consisting of a model L-7100 pump, a Rheodyne model 7125 syringe loading sample injector, a model L-4000 UV-visible light detector (set at 254 nm), and a Model D-2500 chromatointegrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 mm × 4.6 mm; E. Merck, Darmstadt, Germany) was used for chromatographic separation. The gradient elution program began with 50:50 (vol/vol) acetonitrile/water at a flow rate of 1.0 mL/min for 19 minutes, followed by a linear increase to 90:10 acetonitrile:water (1.0 mL/min) during the next 1.0 minutes. The acetonitrile/water mix was decreased to 50:50 (1.0 mL/min) for 10 minutes.

2.7. DNA extraction and PCR amplification of fish species

DNA of suspected fried fish sticks was extracted according to the protocol described in our previous study [5]. The PCR primers CytBL: 5′-CCATCCAACATCTCAGCATGATGAAA-3′ and CytBH: 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ specific for *cyt b* gene of fish were designed and used to amplify a 348-

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bp fragment by PCR [28]. PCR amplification reactions and sequencing for identification of fish species were performed according to the previously described method [7]. In brief, PCR reaction was carried out in a Gene-Amp PCR system 2400 (Perkin-Elmer, Foster City, CA, USA) programmed to perform a denaturation step at 95°C for 10 minutes, followed by 40 cycles consisting of 1 minute at 95°C, 1 minute at 50°C, and 2 minutes at 72°C. The last extension step was extended to 10 minutes at 72°C. PCR products (6 μL) were loaded onto a 2% agarose gel and electrophoresed. The DNA band was excised under UV light and melted in five volumes of Tris-EDTA buffer at 65°C for 5 minutes. Purified DNA PCR products were sequenced at Mission Biotech (Taipei, Taiwan) using the above primers and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer/Applied Biosystems Division) in a ABI PRISM 377-96 DNA sequencer (Perkin-Elmer/Applied Biosystems Division). The sequences were analyzed using the BLAST (National Center for Biotechnology Information (NCBI)) for the identification of fish species.

3. Results and discussion

3.1. *Identification of fish species present in suspected fried fish sticks*

DNA extracts from the suspected fried fish sticks were tested for PCR amplification with CytBL and CytBH primers, which generated a 348-bp fragment. This sequence was submitted to GenBank for accession no. AY187632.1 of *C. chanos* (milkfish) (Fig. 1). Consequently, the species of the suspected fried fish stick was identified as *C. chanos*. Because the suspected fish samples implicated in histamine poisoning have usually undergone significant heating, almost all the proteins in the fish samples are denatured and damaged. Therefore, protein analysis methods for the identification of fish species, such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis, isoelectric focusing, and two-dimensional electrophoresis, are inappropriate, however, PCR and direct sequence analysis of mitochondrial *Cyt b* genes are successful for the identification of fish species. In Taiwan, *Xiphias gladius* (swordfish), *Makaira nigricans* (blue marlin), *Makaira indica* (black marlin), *Istiophorus platypterus* (sailfish), and *Tetrapturus angustirostris* (small marlin) have been identified to cause histamine food-borne poisoning [5–7]. In conclusion, scombroid poisoning linked to the consumption of milkfish has rarely been reported in Taiwan, however, it is important for Taiwanese people to be aware that milkfish sticks can cause histamine poisoning if the fish flesh is contaminated with histamine-forming bacteria and is stored at improper temperatures. Prolific histamine-forming bacteria isolated from fish skin, floor, and fish meat samples in traditional seafood-processing factories demonstrate the potential risk for contamination of fish with these bacteria and greater risk of histamine development [4,6,15]. Therefore, we postulate that suspected milkfish stick samples had been seriously contaminated during fish preparation and processing.

3.2. *Chemical and microbiological qualities of suspected fried fish sticks and commercial raw fish stick samples*

Table 1 presents the values of the pH, salt content, TVBN, APC, TC, and *E. coli* in the suspected fried fish sticks implicated in food-borne poisoning and the 16 raw fish stick samples from retail stores. The values in both suspected fried fish sticks were as follows: pH, 6.15 and 6.08; salt content, 0.85% and

```
                1                                                  50
AY187632.1 CAACCNNACGTATTGANTGTTCTGCNTGCCCAGTGACTATAGGTTCAACG
    Sample CAACCNNACGTATTGANTGTTCTGCNTGCCCAGTGACTATAGGTTCAACG
               51                                                 100
AY187632.1 GCCGCGGTATTTTGACCGTGCAAAGGTAGCGCAATCACTTGTCTCTTAAA
    Sample GCCGCGGTATTTTGACCGTGCAAAGGTAGCGCAATCACTTGTCTCTTAAA
              101                                                 150
AY187632.1 TAGAGACCTGTATGAATGGCACGACGAGGGCTTAACTGTCTCTNCTCCCC
    Sample TAGAGACCTGTATGAATGGCACGACGAGGGCTTAACTGTCTCTNCTCCCC
              151                                                 200
AY187632.1 AGTCAGTGAAATTGATCTGTCCGTGCAGAAGCGGACATTGTACTGCAAGA
    Sample AGTCAGTGAAATTGATCTGTCCGTGCAGAAGCGGACATTGTACTGCAAGA
              201                                                 250
AY187632.1 CGAGAAGACCCTATGGAGCTTTAGACACAAGACCAACCATGTTAAACCAC
    Sample CGAGAAGACCCTATGGAGCTTTAGACACAAGACCAACCATGTTAAACCAC
              251                                                 300
AY187632.1TTAAAACAAAACAAGTGGAAACAGAGNGGTAAACCTTGGTCCGCCTGTCT
    Sample TTAAAACAAAACAAGTGGAAACAGAGNGGTAAACCTTGGTCCGCCTGTCT
              301                                         348
AY187632.1 TCGGTTGGGGCGACCGCGGGGCAAANACAAACCCCCATGTGGAAT
    Sample TCGGTTGGGGCGACCGCGGGGCAAANACAAACCCCCATGTGGAAT
```

Fig. 1 – DNA sequences of 348-bp region of cytochrome *b* gene from suspected fried fish sample and accession number AY187632.1 of *Chanos chanos* (milkfish).

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Table 1 – Values of pH, salt content, APC, TVBN, TC, and *Escherichia coli* in the fried fish sticks implicated in food poisoning, and the 16 raw fish stick samples collected from retail stores.

Sample type, no.	pH	Salt content (%)	TVBN (mg/100 g)	APC (log CFU/g)	TC (MPN/g)	*E. coli* (MPN/g)
Victims' leftover fried fish sticks						
F-1	6.15	0.85	15.09	2.40	<3	<3
F-2	6.08	0.81	15.37	2.35	<3	<3
Retail stores' raw fish stick samples						
R-1	5.57	0.98	28.62	6.42	200	<3
R-2	5.67	1.01	21.99	6.06	2,100	<3
R-3	5.63	1.13	27.16	6.24	200	<3
R-4	5.67	1.09	21.54	6.24	<3	<3
R-5	5.58	1.06	18.61	6.48	420	<3
R-6	5.69	1.02	19.25	6.23	<3	<3
R-7	5.69	1.11	33.95	6.74	1,200	<3
R-8	5.63	1.07	25.59	6.04	750	<3
R-9	5.63	0.90	19.00	6.59	4,600	<3
R-10	5.76	1.18	23.87	5.53	<3	<3
R-11	5.77	1.05	27.37	5.61	<3	<3
R-12	5.70	1.08	21.65	7.03	>24,000	<3
R-13	5.71	1.02	19.97	7.01	11,000	<3
R-14	5.71	1.08	15.56	6.16	1,200	<3
R-15	5.75	1.03	22.23	6.06	350	<3
R-16	5.70	1.11	40.06	6.42	>24,000	<3
Average[a]	5.68 ± 0.06	1.06 ± 0.07	24.15 ± 6.24	6.30 ± 0.42	4,377 ± 8,148	

APC = aerobic plate count; CFU = colony-forming unit; MPN = most probable number; SD = standard deviation; TC = total coliform; TVBN = total volatile basic nitrogen.
[a] Mean ± SD for raw fish stick samples R-1 to R-16 collected from retail stores.

0.81%; TVBN, 15.09 mg/100 g and 15.37 mg/100 g; and APC 2.40 log CFU/g and 2.35 log CFU/g. None of the two fried fish samples contained TC and *E. coli*. In commercial raw fish samples, the levels of pH, salt content, TVBN, APC, and TC ranged from 5.57 to 5.77, 0.90% to 1.18%, 15.56 mg/100 g to 40.06 mg/100 g, 5.53 log CFU/g to 7.03 log CFU/g, and <3 MPN/g to >24,000 MPN/g, respectively. None of those raw fish samples contained *E. coli*. The salt content, TVBN, and APC values of both suspected fried samples were lower than the average values of the 16 raw fish stick samples (1.06%, 24.15 mg/100 g, and 6.3 log CFU/g, respectively; Table 1). Based on the Taiwanese regulatory standard of 6.47 log CFU/g of APC for raw frozen fish [29], 31.3% (5/16) of the commercial raw fish samples were unacceptable (Table 2). Although a Taiwanese regulatory standard of TC for raw frozen fish is not set, 12 of 16 commercial raw fish samples (75%) contained >200 MPN/g of TC (Table 1). Six of 16 commercial raw fish samples (37.5%) contained >25 mg/100 g of histamine, which is the Taiwanese allowable limit for raw frozen fish (Table 2). Concerning the hygienic quality, Hsu et al [15] also reported that dried milkfish products had the unacceptable rates of 46.9% for APC and 68.8% for TVBN.

3.3. *Contents of biogenic amines in suspected fried fish sticks and commercial raw fish stick samples*

The levels of biogenic amines in the suspected fried fish sticks responsible for histamine poisoning illness and the 16 raw fish sticks samples obtained from retail stores are summarized in Table 3. Although eight of the biogenic amines in both suspected fried samples occurred at levels <5.4 mg/100 g, F-1 and F-2 samples contained 235.0 mg/100 g and 86.6 mg/100 g histamine, respectively (Table 3). In most scombroid poisoning cases, histamine levels in fish that caused the illness have been >20 mg/100 g, often >50 mg/100 g [30]. The Centers for Disease Control and Prevention [31] also reported that histamine levels at 20 mg/100 g may be sufficient to cause the symptoms of scombroid poisoning. However, Bartholomev et al [32] demonstrated that histamine at >100 mg/100 g in fish would be toxic and unsafe for human consumption. Thus, the high levels of histamine in the fried fish sticks, along with the allergy-like symptoms that developed in the victims, supported the conclusion that histamine was the causative agent of this food-borne poisoning incident. Various types of fish

Table 2 – Percentage of unacceptability of 16 raw fish stick samples collected from retail stores.

Sample type	No. of sample	Percentage (%) of unacceptability [a]				
		APC	TC	*E. coli*	TVBN	Histamine
Retail store samples	16	31.3 (5/16) [b]	— [c]	0 (0/16)	37.5 (6/16)	25.0 (4/16)

APC = aerobic plate count; CFU = colony-forming unit; MPN = most probable number; TC = total coliform; TVBN = total volatile basic nitrogen.
[a] The regulatory limits of APC, *E. coli*, TVBN, and histamine for raw frozen fish in Taiwan are <6.47 log CFU/g, <10 MPN/g, 25 mg/100 g, and 5.0 mg/100 g, respectively.
[b] The number of unacceptable samples/the number of total samples tested.
[c] TC has no regulatory level.

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Table 3 – Levels of biogenic amines in the fried fish sticks implicated in food poisoning and the 16 raw fish stick samples collected from retail stores.

Sample type, no.	Levels of biogenic amine (mg/100 g)								
	Put	Cad	Try	Phe	Spd	Spm	His	Tyr	Agm
Victims' leftover fried fish sticks									
F-1	0.90	5.25	ND	ND	ND	ND	235.0	0.50	ND
F-2	0.50	1.90	ND	ND	ND	ND	86.6	5.40	ND
Retail stores' raw fish stick samples									
R-1	ND	5.50	ND	ND	ND	ND	7.60	ND	ND
R-2	ND	1.70	ND	ND	ND	ND	1.30	ND	ND
R-3	ND	0.50	ND	ND	ND	ND	4.10	ND	ND
R-4	ND	3.30	ND	ND	ND	8.10	1.50	ND	ND
R-5	0.14	ND	ND	ND	ND	ND	1.40	0.90	ND
R-6	0.15	2.01	ND	ND	ND	ND	0.60	1.70	ND
R-7	0.18	0.32	0.33	0.26	ND	0.21	7.90	1.03	ND
R-8	0.18	0.12	ND	0.17	ND	ND	2.50	0.91	ND
R-9	0.06	ND	ND	ND	ND	ND	0.40	ND	ND
R-10	0.02	0.92	ND	ND	ND	ND	1.00	0.61	ND
R-11	0.02	ND	ND	0.17	ND	ND	6.30	ND	ND
R-12	0.02	ND	ND	ND	ND	ND	0.30	0.68	ND
R-13	ND	3.45	3.17	0.21	ND	2.25	0.70	0.75	ND
R-14	ND	ND	ND	ND	ND	ND	0.70	ND	ND
R-15	0.13	ND	ND	ND	ND	ND	0.30	0.80	ND
R-16	0.14	ND	ND	0.17	ND	ND	9.10	ND	ND
Average [a]	0.10 ± 0.07	1.11 ± 1.66	0.22 ± 0.79	0.06 ± 0.10	ND	0.66 ± 2.06	2.86 ± 3.09	0.46 ± 0.53	ND

Agm = agmatine; Cad = cadaverine; His = histamine; ND = not detected (amine level <0.05 mg/100 g); Phe = 2-phenylethylamine; Put = putrescine; SD = standard deviation; Spd = spermidine; Spm = spermine; Try = tryptamine; Tyr = tyramine.
[a] Mean ± SD for raw fish stick samples R-1 to R-16 collected from retail stores.

implicated in scombroid poisoning have been found to contain high levels of histamine. The histamine content of marlin implicated in a poisoning incident ranged between 93.5 mg/100 g and 276 mg/100 g [33]. The hot-smoked mackerel implicated in a scombrotoxic incident had a histamine content of 270 mg/100 g [34]. In Taiwan, fish implicated in occasional scombroid poisoning incidents [2,3] have included tuna, mackerel, and marlin, and with more frequency recently, sailfish, swordfish, and marlin fillets [5–7]. In a previous study, the high content of histamine at 61.6 mg/100 g in the suspected dried milkfish products could be the etiological factor for this fish-borne poisoning in Taiwan [4]. However, strong evidence exists that biogenic amines such as putrescine, cadaverine, spermine, and spermidine in fish tissue can increase the toxic effects of histamine by inhibiting intestinal histamine-metabolizing enzymes such as diamine oxidase, thereby increasing histamine uptake and liberating endogenous histamine in intestinal fluids [35]. Quality loss and histamine accumulation often occur after the abovementioned species of fish are frozen and then thawed and kept for long periods of time at room temperature before further processing. Because histamine is heat resistant, it can remain intact in canned or cooked fish products [36]. In this case, the suspected fish sticks might have been stored at an improper temperature prior to cooking, allowing formation of histamine by histamine producers.

Except for histamine (2.86 mg/100 g), the average content of various biogenic amines in the 16 commercial raw fish samples was <1.11 mg/100 g (Table 3). Among them, four samples (R-1, R-7, R-11, and R-16) had 7.60 mg/100 g, 7.90 mg/100 g, 6.30 mg/100 g, and 9.10 mg/100 g of histamine, respectively, which are greater than the 5.0 mg/100 g allowable limit suggested by the US Food & Drug Administration (FDA) for scombroid fish and/or products [30]. Therefore, 25% (4/16) of the commercial raw fish samples were unacceptable (Table 2). Recently, we demonstrated that most of the tested dried milkfish products (78.1%) sold in Taiwan had histamine levels greater than the FDA guideline of 5 mg/100 g for scombroid fish and/or products [15]. Based on the finding that higher levels of histamine, APC, TVBN, and TC occurred in some commercial raw fish samples, we postulate that these samples had been seriously contaminated during fish preparation and processing.

3.4. *Isolation and identification of histamine-forming bacteria from commercial raw fish stick samples*

Table 4 lists the identities of 10 histamine-forming bacteria determined by 16S ribosomal DNA sequences, following comparison with reference strains, using NCBI database analysis. The PCR amplicons from strains M1, M2, M6, and M7 had 100% homology with *E. aerogenes*, those from strains M3 and M4 had 100% homology with *M. morganii*, and the PCR amplicon from strain M5 had 99% homology with *Serratia marcescens* (Table 4). The PCR amplicon from strain M8 aligned with *H. alvei* at 99%, that from strain M9 aligned with *E. cloacae* at 100%, and that from strain M10 aligned with *R. ornithinolytica* at 100%. These 10 histamine-forming isolates produced substantial amounts of histamine (373–1261 ppm) in TSBH medium. Some of them also produced different amounts of putrescine, cadaverine, and tyramine through the actions of their respective decarboxylase enzymes on various amino acids that were also present in the culture medium (Table 4).

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Table 4 – Identification of histamine-forming bacteria isolated from the 16 raw fish stick samples collected from retail stores by 16S rDNA, based on the output results from NCBI database analysis, and their production of histamine and other biogenic amines in culture broth.

Strain	Organism identified	% identity	Sample no. and histamine content (mg/100 g) in fish sample	GenBank accession no.	His (ppm)	Put (ppm)	Cad (ppm)	Tyr (ppm)
M1	*E. aerogenes*	100	R-1, 7.6	CP002824.1	1256	ND	162	ND
M2	*E. aerogenes*	100	R-1, 7.6	CP002824.1	421	ND	136	8.3
M3	*M. morganii*	100	R-3, 4.1	KF471513.1	961	ND	153	3.9
M4	*M. morganii*	99	R-3, 4.1	HQ169125.1	1173	ND	110	ND
M5	*S. marcescens*	99	R-7, 7.9	KC206270.1	838	ND	136	ND
M6	*E. aerogenes*	100	R-7, 7.9	AY825036.1	373	ND	110	ND
M7	*E. aerogenes*	100	R-7, 7.9	AB844449.1	448	ND	129	ND
M8	*H. alvei*	99	R-8, 2.5	AY253922.1	1261	ND	332	ND
M9	*E. cloacae*	100	R-11, 6.3	KC999878.1	1260	44.8	ND	11.0
M10	*R. ornithinolytica*	100	R-16, 9.1	KC456519.1	1028	83.3	124	ND

Cad = cadaverine; His = histamine; ND = not detected (amine level <0.05 ppm); Put = putrescine; Tyr = tyramine; NCBI = National Center for Biotechnology Information.

In this study, all histamine-forming isolates belonged to Enterobacteriaceae, which are generally thought to be the primary cause of histamine development in scombroid fish [12]. Among them, *E. aerogenes* (4 strains), *E. cloacae* (1 strain) and *R. ornithinolytica* (1 strain) produced 373–1260 ppm of histamine in TSBH (Table 4), accounting for 60% of histamine-forming isolates. *E. aerogenes* and *R. ornithinolytica* were the prolific histamine formers most frequently reported in tuna [37], albacore [38], and sailfish [18]. The enteric bacteria *E. aerogenes* and *R. ornithinolytica*, isolated from dried milkfish implicated in a food-borne poisoning incident, were reported to be potent histamine producers capable of producing 500 ppm of histamine in TSBH at 35°C for 24 hours [4,15]. In previous studies, *E. aerogenes* and *R. ornithinolytica* that were isolated from tuna dumpling and tuna sandwich products were also identified as prolific histamine formers [16,17]. Recently, *E. aerogenes* and *R. ornithinolytica* isolated from the suspected raw striped marlin fillets were identified as prolific histamine formers, able to produce >129 ppm of histamine in TSBH medium [39]. In this study, four histamine-producing bacterial strains isolated from samples R-1 (7.6 mg/100 g histamine) and R-7 (7.9 mg/100 g histamine), which were capable of producing 373–1256 ppm of histamine in TSBH, were identified as *E. aerogenes*, while the *E. cloacae* strain M9 and *R. ornithinolytica* strain M10 isolated from samples R-11 (6.3 mg/100 g histamine) and R-16 (9.1 mg/100 g histamine) were also potent histamine formers and produced 1260 ppm and 1028 ppm of histamine in TSBH, respectively (Table 4). Therefore, we conclude that *E. aerogenes*, *E. cloacae*, and *R. ornithinolytica* were the bacteria that produced higher levels of histamine, which in turn were responsible for the higher histamine contents (>5.0 mg/100 g) of those raw fish samples.

Note that the *M. morganii* strains M3 and M4, isolated from the raw fish sample R-3 (4.1 mg/100 g histamine) in this study, were prolific histamine formers, and they produced 961 ppm and 1173 ppm of histamine in TSBH, respectively (Table 4). Moreover, the *H. alvei* strain M8 isolated from samples R-8 (2.5 mg/100 g histamine) was also a potent histamine former and produced 838 ppm of histamine in TSBH. *H. alvei*, *M. morganii*, and *K. pneumoniae* have been implicated as causative organisms in the formation of toxicologically significant levels of histamine in outbreaks of scombroid fish poisoning [11]. Among them, *M. morganii* has consistently been shown to form high levels of histamine (>1000 ppm) in culture broth and isolated from spoiled fish stored above 15°C [37]. The *S. marcescens* strain M5 isolated from sample R-7 (7.9 mg/100 g) in this study was a prolific histamine former, with the ability to produce 838 ppm histamine in TSBH (Table 4). However, *S. marcescens*, *Serratia plymuthica*, and *S. fonticola* have been isolated from tuna as weak histamine formers, producing 5.5–134.8 ppm of histamine in culture broth [37]. *S. marcescens* isolated from tuna dumpling products implicated in a food-borne poisoning incident in Taiwan were also shown to be weak histamine formers [2].

4. Conclusion

The high content of histamine (86.6 mg/100 g and 235.0 mg/100 g) detected in both the suspected fried fish sticks could be the etiological factor for the fish-borne poisoning examined in this study. The species of the suspected fish stick was identified as *C. chanos* (milkfish). In addition, this study also analyzed 16 raw fish stick samples sold in southern Taiwan and showed that 31.3% and 37.5% of samples had APC and TVBN levels greater than the Taiwanese regulatory limit of 6.47 log CFU/g and 25 mg/100 g, respectively. The histamine contents in 25% of commercial raw fish samples exceeded the 5 mg/100 g FDA guideline value. Ten isolates isolated from commercial raw samples were proved to be prolific histamine formers with the ability to produce 373–1261 ppm of histamine in TSBH.

Conflicts of interest

All authors declare no conflicts of interest.

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