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A Survey of *Vibrio parahaemolyticus* in Imported Fishery Products in the Kaohsiung Area

Abstract

The purpose of this study is to investigate the existence and distribution of *Vibrio parahaymolyticus* in the fishery products imported from different Asian countries. In addition, toxigenic genes related to *Vibrio paraphaymolyticus,* such as thermostable direct hemolysin (TDH) and thermostable direct related hemolysin (TRH) are tested. Between July 1997 and April 1998, specimens collected from 686 fishery products imported through the Kaohsiung International Airport were tested of *Vibrio parahaymolyticus* and its toxigenic genes. *Vibrio parahaemolyticus* was isolated in 45.9% of the specimens, 81.3% in green crabs imported from Thailand, 75.8% in live shrimps imported from Thailand, and 71.1% in green crabs imported from Hong Kong. Products of different countries of origin showed statistically significant differences ($p<0.01$). The results of statistical analyses indicated that the detection rate was higher in the crustaceans (49.4%) than in the non-crustaceans (37.4%). Detection rates by month ranged from 36.7% to 51.1%, showing little variation. Testing for toxigenic genes of *Vibrio parahemolyticus* such as TDH and TRH by polymerase chain reaction (PCR) in fishery products gave negative responses in 187 product items. Though toxigenic genes in the present study were negative, reports are that Kanagawa-phenomenon negative strains can also generate cellular toxicity in animal experiments to cause deaths in rats and induce accumulation of ileum tract fluids in rabbits. The high detection rate of *Vibrio parahaemolyticus* in imported fishery products is a warning. Appropriate cooking and freezing of fishery products are most important methods in the prevention of *Vibrio parahaemolyticus* poisoning.

Introduction

Vibrio parahaemolyticus is one of the major pathogens of food poisoning in Taiwan. Between 1992 and 1997, *Vibrio parahaemolyticus* had been the leading cause of food poisoning outbreaks, increasing from 20 outbreaks in 1992 to 106 in 1997[1]. *Vibrio parahaemolyticus* is also one of the major causes of food poisoning outbreaks in school lunch programs and outdoor catering cooking[2,3]. For those tourists returning from the Southeast Asian countries and suffering from gastroenteritis, *Vibrio parahaemolyticus* is often detected from their stool specimens[4,5]. The major reasons for this pathogenic agent to cause outbreaks are cross-contamination of raw and cooked food, raw food not well cooked, poor personal hygiene, and inadequate preservation of food[2,6].

With improvement in the standards of living and changes in food habits in the recent years, more fishery products have been imported from abroad. For instance, in 1993, 159,097,183 kilograms in 39,562 batches; in 1994, 168,588,283 kilograms in 43,516 batches; in 1995, 187,389,864 kilograms in 48,774 batches; in 1996, 184,960,913 kilograms in 51,989 batches[7]; and in 1997, 271,364,261 kilograms of fishery products in 52,177 batches[8] were imported. Of the 1997 importation, 31,768,194 kilograms of live fishery products were handled by the Taipei Quarantine Station, 5,881,856 kilograms by the Kaohsiung 2nd Quarantine Station, and the rest were frozen products. The amount of fishery products imported is large, quarantine surveillance is most necessary. However, no studies on the prevalence of *Vibrio parahaemolyticus* in imported fishery products have yet been conducted to understand the existence and distribution of *Vibrio parahaemolyticus* in imported fishery products. The present survey was to present some preliminary descriptive statistics on *Vibrio parahaemolyticus* detected in fishery products by country of origin and food items. Epidemiological investigations have found that 88-96% of clinically isolated *Vibrio parahaemolyticus* will produce thermostable direct hemolysin (TDH), whereas only 1-2% of strains isolated from environment will produce TDH[9,10,11]. TDH is considered highly associated with the pathogenicity of *Vibrio parahaemolyticus* and is the cause of diarrhea. The present survey applied the polymerase chain reaction (PCR) to detect TDH [12] and the thermostable direct related hemolysin (TRH) [13] of *Vibrio parahaemolyticus* in fishery products. Distribution of *Vibrio parahaemolyticus* in fishery products by month and by season was also reviewed for reference in the formation of disease control policies.

Materials and Methods

1.Sources of Specimens

Betweem July 1997 and April 1998, 686 specimens of live fishery products imported through the Kaohsiung International Airport were collected. They included: 126 crabs, 59 lobsters, 95 mantis shrimps, and 106 snails from Vietnam; 114 live green crabs from Hong Kong; 32 live green crabs and 62 shrimps from Thailand; and 92 live fish from Indonesia.

2.Culture Agars

For multiplication, 3% alkaline peptone-salt-broth (APS), pH 8.2-8.4, was used. For selective isolation, thiosulfate-citrate-bile-salts (TCBS) sucrose agar was used. For screening, triple sugar iron agar (TSI) was used. For purification, 3% NaCl nutrient agar was used.

3.Culture Method

1-3 specimens were collected from each batch of crabs, lobsters, mantis shrimps, and live green crabs; 20-30 snails, 15-20 shrimps, and one live fish was collected from each batch. The specimens were placed in sterile bags filled with distilled water with 1% NaCl, diluted, and added 3% APS for multiplication under 37 ℃ for 6-7 hours. They were inoculated on TCBS agars and kept overnight at 37 ℃.

4.Assessment of Strains

Colonies at a diameter of 2-3 mm with greenish or bluish spots at the center were considered *Vibrio parahaemolyticus*. One colony was picked up and inoculated on TSI containing 1% NaCl for overnight at 37 ℃. Colonies of yellowish bottom, reddish slant, no gas-producing, and no sulfur hydride producing were selected and inoculated on 3% NaCl nutrient agar for 12-18 hours under 37 ℃. Lastly, API 20E (bioMerieux, France) was used for the assessment of strains.

5.Testing for TDH and TRH of *Vibrio parahaemolyticus* by PCR

1) Oligonucleotide primers

Based on known sequences of tdh and trh[12,13], the following oligonueleotide primers were synthesized:

tdh: 5'-GTACCGATATTTTGCAAA-3' and 5'-ATGTTGAAGCTGTACTTGA-3'

trh: 5'-CTCTACTTTGCTTTCAGT-3' and 5'-AATATTATGGAGTTTCAT-3'.

The length of DNA was 382 and 460 bp.

2) Preparing PCR Plates

The strains were cultured in 1% NaCl nutrient agar overnight under 37 ℃. Some colonies were placed in 300 μl TE buffer (Tris HCl 10 mM, EDTA disodium salt 1 mM) containing 0.1% sodium dodecyl sulfate, heated to destroy cell walls, centrifuged at 3000 rpm, and then placed in clean tube for keeping at -20 ℃.

3) PCR Procedures

The reaction solution included 3 μl PCR plate, 3 μl 10x PCR buffer (10 mM MgCl2, 500 mM KCl, 100 mM Tris HCl, pH8.3), 10 μm tdh primer 0.9 μl, 10 μm trh primer 0.9 μl, dNTP 2.4 μl, Taq polymerase 0.15 μl, ddH2O 20 μl, and finally, mineral oil. The PCR reaction conditions were one minute under 94 ℃, one minute under 48 ℃, and one minute under 72 ℃ for 40 rounds, and finally for 5 minutes under 72 ℃.

4) Electrophoresis

1 μl loading buffer was added to 5 μl PCR product, placed in 2% agarose electrode under 100 volts. The electrode was then placed in 0.5 μg/ml

ethidium bromide for dyeing for 10-15 minutes and studied under UV light. By the position of the marker, the existence of tdh and/or trh was assessed.

5) Statistical Analysis

Epi Info version 6.0 was used for statistical analysis. χ^2 test was used for testing. A p value of less than 0.01 was accepted as statistically significant.

Results and Discussion

1.Distribution of *Vibrio parahaemolyticus* in Different Fishery Products

Of the 686 specimens collected from imported fishery products, 315 were detected of *Vibrio parahaemolyticus*, giving a detection rate of 45.9%. *Vibrio parahaemolyticus* was detected highest in green crabs (Thailand 81.3%, Hong Kong 71.1%), 73.3% in live green crabs, 44.3% in snails, 44.1% in live lobsters, 32.5% in crabs, 29.7% in live fish, and 21.1% in mantis shrimps (see Table 1). In 1982-1983, Sarker et al. found that *Vibrio parahaemolyticus* in live fish was detected more in feces (74.4%), gills (46.4%), and body surface (26.7%)[14]. Specimens for the present survey were collected from gills. Whether the site of specimen collection was a reason of the low detection rate remained to be studied. When all specimens were classified into crustacean (live shrimp, live green crab, crab, live mantis shrimp, live lobster) and non-crustacean (fish and snail), the detection rate was higher (49.4%) in the crustaceans than in the non-crustaceans (37.4%), the difference being statistically significant (Table 2). Literature shows that the crustacean fishery products contain chitin, and chitin is of affinity to *Vibrio parahaemolyticus*[15]. This could be the reason why more *Vibrio parahaemolyticus* was detected in the crustaceans . By countries of origin, green crabs imported from Thailand had a higher detection rate (81.3%) than those imported from Hong Kong (71.1%), the difference though not statistically significant. Green crabs had higher detection rate (73.3%) than crabs (32.5%), the difference being statistically significant. Shih et al., in their survey of 39 shell fish specimens collected from markets in the Taipei area between September 1994 and January 1995, detected *Vibrio parahaemolyticus* in 9 of them, at a detection rate of 23.1%[16]. Wong et al., in a survey of Taiwan fishery products in 1990, detected *Vibrio parahaemolyticus* in 70.2% of shrimps and 41.7% of green crabs[17], the detection rates were lower than those of imported fishery products.

2.Distribution of *Vibrio parahaemolyticus* by Month

By month (Table 3), the detection rate was the highest in January 1998 at 53.6%, and the lowest in December 1997 at 36.7%. Detection rates by month showed little variation. *Vibrio parahaemolyticus* grows better under 37 ℃, and multiplies rapidly under room temperature. The products came from the tropic and sub-tropic Southeast Asian countries, the temperatures vary little by month. This probably was the reason why there was little variation in the detection rates by month.

3.Distribution of *Vibrio parahaemolyticus* by Season

By season (Figure 1), green crabs had higher detection rate in spring and winter (81.6% and 81.5%); mantis shrimps in summer (37.5%); live fish in spring and winter (40.0% and 37.0%); lobsters in winter (60.0%); crabs in autumn (48.3%); shrimps in summer, spring and winter (100%, 81.5% and 81.3%); and snails in summer and autumn (63.3% and 56.7%). Different products had different detection rates by season. Whether this was due to different countries of origin or other factors remains to be studied.

4.The Biochemical Characteristics of *Vibrio parahaemolyticus*

In 1985, Timothyl et al. tested 60 members of the family *Vibrionaceae*[18]. In the 21 API 20E biochemical tests, the typical reactions of *Vibrio parahaemolyticus* corresponded to the API 20E kits. The biochemical reactions of the 315 *Vibrio parahaemolyticus* strains in the present survey corresponded approximately to findings elsewhere. In the present survey, when using API 20E kits for reading, in some kits, the ortho-nitro-phenyl-galactoside (ONPG) reactions were slightly yellowish, though turned colorless after repeated tests. This perhaps was a problem of the kits. 5% of *Vibrio parahaemolyticus* showed positive ONPG reaction[19]. It was perhaps confused with *V. vulnificus* in the reading. Massad and Oliver pointed out in 1987 that *V. parahaemolyticus* and *V. vulnificus* could be easily distinguished by using cellubiose-polymysin B-colostin (CPC) agar under 40 ℃[20]. In the future testing of fishery products, CPC agar can be used for the confirmation of these two agents.

5.PCR Testing for Toxigenic Genes

187 randomly selected *Vibrio parahaemolyticus* strains were tested for tdh and trh, and both were found negative. Two clinical strains were found tdh positive and trh negative when they were tested with PCR. Reports are that urea hydrolysis positive strains can be used as a trh possessing marker[21]. In 1995, Suthienkul et al. detected in 489 clinical specimens 37 urea hydrolysis positive, at a rate of 7.5%, all possessing trh[21]. In the present survey, only three specimens were found urea hydrolysis positive, at a rate of 0 95%, and not possessing trh. It could be that fewer strains of imported fishery products were urea hydrolysis positive than clinical strains and that less of them possessed trh.

Conclusion and Recommendations

1. In general, clinical specimens collected from patients in the acute stage are sent to laboratories for testing. For the detection of *Vibrio parahaemolyticus*, the specimens are inoculated directly on TCBS with good result. Reports are not in agreement as to the time required for the culturing of environmental specimens. For instance, the multiplication agar APS is suggested to be placed in glucose-salt-teepol broth under 35-37 ℃ for 16-18 hours[16]; whereas the multiplication agar APW is to be placed under 37 ℃ for 7-8 hours[17]; and the multiplication agar APW under 37 ℃ for 15-18 hours[22]. The time chosen in the present survey for the multiplication agar was 16-18 hours. The detection rate was rather low at 12% (7/60). By adjusting the time, 6-8 hours was found most adequate for the

multiplication of *Vibrio parahaemolyticus*. It is most important to control both the time and temperature in the testing of pathogenic agents..

2. Taiwan and Japan had the greatest number of *Vibrio parahaemolyticus* outbreaks during 1973-87, this pathogen accounting for 57% and 32% of the outbreaks respectively[22]. Epidemiological analyses show 88-96% of TDH in clinically isolated *Vibrio parahaemolyticus*, though only 1-2% of TDH in environmental strains[23,24]. In the present survey, PCR was applied to detect 187 fishery products to find both tdh and trh negative. Though reports are that Kanagawa negative strains can induce gastroenteritis, Wong et al. in 1992 and 1993 confirmed that some Kanagawa negative strains could induce cytotonic and cytotoxic toxicity in the ovary cells of Chinese hamsters, and producing lethality in mice[17,25]. In 1991, Honda et al. discovered Vp-TDH/I in Kanagawa negative strains. The Vp-TDH/I was said to induce accumulation of ileum fluids in rabbits. This could account for *Vibrio parahaemolyticus* Kanagawa negative strains inducing gastroenteritis[26]. The detection rate of *Vibrio parahaemolyticus* was as high as 45.9% in the present survey, though both tdh and trh were negative, the pathogenicity of *Vibrio parahaemolyticus* should not be overlooked. Sanitary control of imported fishery products should still be a priority.
3. The serotype of the *Vibrio parahaemolyticus* outbreaks in Taiwan in 1997 was primarily K6[2,3] . In 1997, of the 12 strains collected from passengers with gastroenteritis entering from Kaohsiung, 9 were found to be K6 (75%). Whether *Vibrio parahaemolyticus* outbreaks in the Southeast Asian countries originate from the same source requires further study.
4. Although the detection rate of *Vibrio parahaemolyticus* in the present survey was as high as 45.9%, and the toxigenic genes were negative, the fact that *Vibrio parahaemolyticus* is not a notifiable nor a reportable disease in Taiwan, no quarantine measures can be taken against fishery products even if toxigenic strains are found on them. *Vibrio parahaemolyticus* is everywhere along coastal lines and in fishery products. The prevention of food poisoning requires the joint efforts of the consumers, the industries and the government. Effective preventive measures are: clean food, utensils and refrigerators, personal hygiene and environmental sanitation, quick processing of raw food, separation of raw and cooked food to avoid cross contamination, keeping food in refrigerator as low temperature inhibits growth of *Vibrio parahaemolyticus*, heating food before eating (*Vibrio parahaemolyticus* is killed in 15 minutes under 60 ℃). This survey should alert all on the issue of food poisoning and the importance of food sanitation, and thus to take precautions.

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Table 1. Detection Rates, Risk Ratio, and its 95% Confidence Interval of *Vibrio parahaemolyticus* in the Imported Fishery Products, Kaohsiung

Country	Products	No. #	Positive	Detection Rate (%)	RR	95% CI
Vietnam		386	134	34.7	0.56*	0.5-0.7
	Crab	126	41	32.5	0.89*	0.8-1.0
	Lobster	59	26	44.1	0.99	1.0
	Mantis	95	20	21.1	0.85*	0.8-0.9
	Snail	106	47	44.3	0.99	0.9-1.1
Hong Kong	Green crab	114	81	71.1	1.23*	1.1-1.3
Thailand		94	73	77.7	1.23*	1.2-1.3
	Green crab	32	26	81.3	1.07*	1.0-1.1
	Shrimp	62	47	75.8	1.13*	1.1-1.2
Indonesia	Fish	92	27	29.4	0.90*	0.9-1.0
Total		686	315	45.9	1.00	---

Specimens collected between July 1997 and April 1998.
95% CI: 95% confidence interval; RR: risk ratio
*P<0.01

Table 2. Detection Rates, Risk Ratio, and its 95% Confidence Interval of *Vibrio parahaemolyticus* in the Imported Crustaceans and Non-crustaceans, Kaohsiung

Category	No. #	Positive	Detection Rate (%)	RR	95% CI
Crustacean	488	241	49.4	1.42*	1.1-1.8
Non-crustacean	198	74	37.4	1.00	---

Specimens collected between July 1997 and April 1998.
Crustacean: lobster, mantis shrimp, green crab, live shrimp, crab
Non-crustacean: live fish and snail

Table 3. Detection Rates, Risk Ratio, and its 95% Confidence Interval of *Vibrio parahaemolyticus* by Month

Year	Month	No. #	Positive	Detection Rate (%)	RR	95% CI
1997	July	67	30	44.9	1.00	1.0-1.1
	Aug	61	29	47.5	1.01	1.0-1.1
	Sept	66	27	40.9	0.98	0.9-1.0
	Oct	64	32	50.0	1.02	1.0-1.1
	Nov	80	39	48.8	1.02	1.0-1.1
	Dec	79	29	36.7	0.95	0.9-1.0
1998	Jan	84	45	53.6	1.04	1.0-1.1
	Feb	94	48	51.1	1.03	1.0-1.1
	Mar	23	10	43.5	1.00	1.0
	Apr	68	26	38.2	0.92	0.9-1.0
Total		686	315	45.9	1.00	---

Specimens collected between July 1999 and April 1998.

Figure 1. Detection Rates of *Vibrio parahaemolyticus* by Season

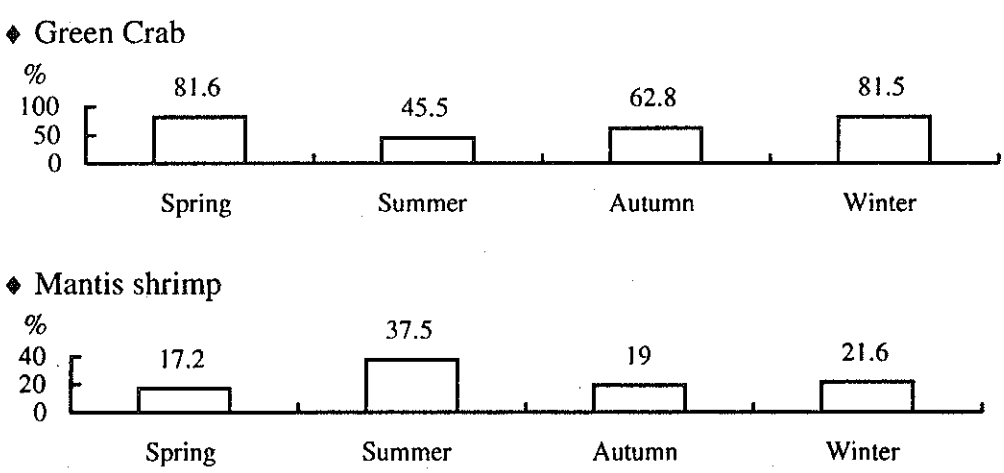

Figure 1. Detection Rates of *Vibrio parahaemolyticus* by Season (Continue)