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Full Paper

Occurrence of potentially pathogenic *Vibrio* species in raw, processed, and ready-to-eat seafood and seafood products

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Abstract: This study investigated the occurrence (by means of the presence-absence test) and level (by means of a plate count technique) of selected potentially pathogenic *Vibrio* species in processed and ready-to-eat seafood, and some raw seafood normally used as raw materials or ingredients in these products, that were commercially available in Chiang Mai, Thailand. The level of *Vibrio* in raw seafood was found to range from 50 to 10^4 cfu/g. *V. alginolyticus* was the most frequently found species, followed by *V. parahaemolyticus*, *V. cholerae*, *V. mimicus*, and *V. vulnificus*, in that order. Processed and ready-to-eat products were contaminated with at least one of the potentially pathogenic vibrios at significant frequencies (25 and 17.5 % of samples, respectively), with the level as high as 10^3 to 10^4 per gram in some samples. Incidences of vibrios revealed by the presence-absence test were significantly higher than those revealed by the plate count assay. These data point to the hazard potential relating to *Vibrio* in processed and ready-to-eat seafood and the need to strictly apply preventive measures against *Vibrio* gastroenteritis through consumption of these food products. They also suggest that analytical methods used in food safety evaluation in relation to potentially hazardous *Vibrio* species should be carefully considered.

Keywords: *Vibrio* species, food safety, seafood, seafood products, ready-to-eat seafood

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Introduction

Vibrio species, Gram negative rod- (or curved rod-) shaped bacteria, are known to occur naturally in marine and freshwater environments and thus are commonly associated with seafood and/or food of freshwater origin [1-3]. Many species can cause gastrointestinal diseases. *V. parahaemolyticus* has been frequently involved in outbreaks of foodborne diseases worldwide [4,5]. *V. cholerae* also constitutes a very important risk. The serogroups O1, O139, and O141 cause cholera, while other serogroups can cause less severe diarrhea [5-7]. *V. vulnificus* is another organism of great concern in seafood safety due to the severity of the disease and the high mortality rate it can cause [1,8-9]. Other species that have been increasingly recognised as food pathogens in recent years are *V. mimicus* and *V. alginolyticus*. *V. mimicus* has genetic and many biochemical similarities to *V. cholerae* [10,11], and its pathogenicity involves several toxins including that of *V. cholerae* [12]. Many foodborne outbreak cases involving *V. mimicus* have been reported [13-15]. *V. alginolyticus* is one of the most common *Vibrio* species occurring in marine environments and seafood [3,16-19]. This species is an opportunistic pathogen [20,21] and its pathogenicity is thought to be similar to that of *V. parahaemolyticus* [22].

The occurrence of *Vibrio* spp. in raw seafood is common, especially seafood from regions with temperate climates around the world, from both natural and farm environments, and in seafood of all types [16-19,23-27]. However, most surveys are qualitative, which causes difficulties in evaluating the risks relating to *Vibrio* spp. in raw seafood. The level of *Vibrio* spp. in raw seafood can also affect survival of the organisms through processing. For processed and ready-to-eat seafood (including ready-to-eat products that are intended for raw consumption, such as raw oyster [28,29]), the presence and level of *Vibrio* spp. has a direct impact on food safety. Cases of foodborne outbreaks resulting from consumption of ready-to-eat seafood dishes, especially those supplied by food catering/food service establishments, continually occur [30-34]. Nevertheless, reports on occurrence of *Vibrio* spp. in processed and ready-to-eat seafood are scarcely available. A few examples are the incidence of *V. parahaemolyticus* in smoked fish [35] and cooked crayfish [36].

The scarce availability of quantitative data for *Vibrio* spp. in seafood and of information about the occurrence of *Vibrio* spp. in processed and ready-to-eat seafood has set the interest for this study. We aim to examine potentially pathogenic species listed above in raw, processed, and ready-to-eat seafood and seafood products by means of qualitative and quantitative methods. Data obtained from this study should benefit food catering/food service establishments, food safety-related personnel and authority, and those involved in food industry.

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Materials and Methods

Food samples

A total of 118 seafood samples or samples containing seafood were randomly collected in Chiang Mai, Thailand. These samples were commercially available in food markets or supermarkets, or prepared by food catering/food service establishments. They consisted of 39 raw seafood samples (squids, crustaceans, fish and shellfish), 16 industrial-scale processed seafood products (seasticks, fish/shrimp balls, fish noodles, fish tofu, squid rolls), and 63 ready-to-eat seafood dishes (sushi-type meals; grilled, fried, steamed, shortly boiled seafood; and seafood-containing dishes prepared by various cooking methods).

Preparation of food samples for analysis of Vibrio *spp.*

Food samples were transferred to the laboratory in closed, sterile containers under cooled conditions. A sample was cut aseptically into small pieces (approx. 0.5-1.0 × 0.5-1.0 cm) and prepared in two separate 25-gram portions for the qualitative (presence-absence) and quantitative (plate count) analyses. For shellfish, the shells were separated and only the flesh was used in analysis. For large-size seafood such as crab or fish, pieces from all different parts were taken for a sample. The food samples were analysed within 2 hours after collection.

Presence-absence analysis and enumeration of Vibrio *spp. in seafood*

Analysis of *Vibrio* spp. (*V. cholerae, V. parahaemolyticus, V. mimicus, V. vulnificus* and *V. alginolyticus*) in seafood was carried out using a method modified from that described in the Bacteriological Analytical Manual [37]. In brief, for presence-absence analysis, a 25-gram portion of a food sample was homogenised in 225 ml of alkaline peptone water (APW, prepared from bacteriological peptone supplied by Hi Media, India), for 1 min using a food homogeniser (Seward Stomacher 400, Brinkmann, Canada). The homogenate was then incubated at 37 ºC for 18 h. This was then transferred to streak on thiosulphate-citrate-bile salt-sucrose (TCBS, Hi Media, India) agar plate, followed by incubation at 37 ºC for 18-24 h. For enumeration, the other 25-gram portion was homogenised in 0.1 % peptone water in the same manner, giving a 10^{-1} dilution, which was further diluted in a 10-fold series until desired dilutions were obtained. The homogenate (0.1 ml) was then surface-spread on TCBS agar plates in duplicate. The inoculated TCBS plates were incubated at 37 ºC for 18-24 h, and a presumptive count was made for each colony type. For both procedures, at least five representative colonies (or all colonies if less than five were recovered) of each colony type were collected and subjected to biochemical tests (sodium chloride tolerance (0-10 %) and lactose utilisation), leading to differentiation of *Vibrio* species according to the species characteristics of human pathogenic Vibrionaceae commonly encountered in seafood listed in the Bacteriological Analytical Manual [37]. The bacterial isolates were maintained on trypticase soya agar (TSA, Hi

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Media, India) slant containing 1.0 % sodium chloride at 4 ºC or as stab cultures in the same medium at room temperature.

Reporting the occurrence and level of Vibrio *spp.*

The results from the qualitative analysis for each *Vibrio* spp. in a food sample was reported as being present or absent in 25 grams of food. From the quantitative analysis, the level of each *Vibrio* spp. in colony forming units per gram (cfu/g) was deduced as followed.

$$\text{Level of a certain } \textit{Vibrio} \text{ species (cfu/g)} = \frac{(R2/R1)\times T}{W}$$

where: R1 = number of representative presumptive colonies (cfu) sampled for analysis
R2 = number of representative colonies (cfu) identified as a certain species
T = Total number (average from duplicates) of presumptive colonies (cfu) on TCBS agar plates of selected dilution
W = weight (gram) of food sample in analytical volume of food homogenate drawn from the dilution in which enumeration was performed

Results and Discussion

The qualitative (presence–absence test) and quantitative (plate count) methods were used in parallel in order to obtain the advantages offered by both. The presence–absence test is more sensitive in revealing the presence of the organisms while the plate count method gives the level of contamination which is more closely related to illnesses potentially caused by *Vibrio* species. The analysis of seafood or seafood-containing samples by the presence-absence test and/or plate count revealed contamination of different potentially pathogenic *Vibrio* spp. in all seafood categories (raw, processed, and ready-to-eat).

Contamination of raw seafood by *V. alginolyticus* was most frequent (61.5 %), followed by *V. parahaemolyticus* (43.6 %), *V. cholerae* (35.9 %), *V. mimicus* (23.1 %), and *V. vulnificus* (2.6 %). The level of contamination ranged from 50 to 4.5×10^4 per gram (Table 1). Since *Vibrio* spp. can occur naturally in an aquatic environment, the presence of these organisms in raw seafood may be expected [38,39]. However, the high level (10^3-10^4 per gram) of *Vibrio* spp. in some samples of raw seafood may indicate inadequate control in storage temperature from the time of harvesting, and this level is regarded as unsatisfactory by some food criteria [39]. Furthermore, the high level (up to 10^4 per gram) of *V. parahaemolyticus* (such as that found in clam, Table 1) is regarded as potentially hazardous [39], considering the possibility of the contaminant strain(s) being pathogenic. These potentially pathogenic vibrios would also have an impact on safety of processed/cooked ready-to-eat food if they survive

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insufficient processing/cooking conditions, or they could be an important source of recontamination after processing.

Table 1. Levels of contamination of potentially pathogenic *Vibrio* spp. in raw seafood

Seafood group	Level of contamination by potentially pathogenic species				
	V. alginolyticus	*V. cholerae*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*
squid	1.0×10^{2}-3.0×10^{4}	3.0×10^{3}-7.0×10^{3}	1.0×10^{2}-9.5×10^{3}	1.0×10^{3}-2.0×10^{3}	1.0×10^{2}
prawn, shrimp, lobster	1.5×10^{2}-1.0×10^{3}	1.8×10^{3}-5.6×10^{3}	not detected	5.0×10^{1}-4.0×10^{2}	not detected
shellfish (clam, mussel, oyster)	1.5×10^{2}-4.5×10^{4}	3.9×10^{3}	1.0×10^{3}-3.3×10^{4}	1.0×10^{2}-1.3×10^{4}	not detected
crab	1.3×10^{3}-2.8×10^{3}	1.6×10^{4}	1.0×10^{2}	1.8×10^{3}-6.7×10^{3}	not detected
fish	6.7×10^{2}-3.5×10^{3}	3.3×10^{2}	2.0×10^{2}	1.1×10^{3}	not detected

Contamination of pathogenic *Vibrio* spp. in industrially processed seafood products and ready-to-eat seafood dishes is demonstrated in Table 2. The summary of the overall positive results obtained by the presence-absence and plate count analyses are given in Table 3.

From Tables 2 and 3, industrially processed and ready-to-eat seafood samples were contaminated with the potentially pathogenic *Vibrio* spp. at significant frequencies. The presence of *V. cholerae* and *V. parahaemolyticus* (as well as other vibrios) in industrially processed and ready-to-eat foods is contrary to what is expected [39-41]. This also applies to other vibrios. Industrially processed seafood products, such as fish/shrimp balls and sea sticks, generally are passed through a pasteurisation process, which should eliminate all non-spore-forming microorganisms. As for the ready-to-eat seafood dishes examined, most were cooked dishes, except one that was intended for raw consumption (prawn in fish sauce). The occurrence of *Vibrio* spp. in processed and cooked ready-to-eat seafood indicated insufficient processing or post-process contamination.

As summarised in Table 3, the proportion of samples contaminated with *Vibrio* spp. as determined by the presence-absence and the plate count method was significantly different. The presence-absence analysis was more sensitive, revealing more positive samples than the plate count analysis, as expected. This stresses the importance, even when quantitative data are required, of applying the presence-absence analysis parallel to enumeration in examining vibrios in seafood for compliance with a zero-tolerant standard. This is crucial, especially when analysing processed food in which low number of organisms are expected, food which has potential to allow low level of *Vibrio*

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contaminants to multiply, food prepared for the immunocompromised group, and food suspected of containing *Vibrio* spp. of severe hazard category or those which have a low infectious dose.

Table 2. Occurrence and level of *Vibrio* spp. in raw seafood, industrially processed seafood products, and ready-to-eat seafood dishes

Sample category	Food sample contaminated with potentially pathogenic *Vibrio* spp.	*Vibrio* species isolated	Occurrence of *Vibrio* spp. in 25 grams of food [a]	Level of *Vibrio* spp. (cfu/g) [b]
industrially processed products	fish balls	*V. cholerae*	P	-
		V. mimicus	P	-
	sea sticks	*V. mimicus*	P	-
	fish balls	*V. cholerae*	P	-
	shrimp balls	*V. parahaemolyticus*	P	-
ready-to-eat seafood dishes	mixed seafood salad	*V. alginolyticus*	P	-
	spicy mackerel salad	*V. alginolyticus*	P	2.02×10^{4}
	prawn salad in lime dressing	*V. mimicus*	P	1.1×10^{3}
		V. cholerae	P	1.0×10^{2}
	steamed seafood in coconut sauce	*V. cholerae*	P	-
	deep-fried battered squid	*V. cholerae*	P	-
		V. mimicus	P	-
	steamed squid in lime sauce	*V. cholerae*	P	-
		V. mimicus	P	-
	squid salad with lime dressing	*V. mimicus*	P	-
	stir-fried prawn with black pepper	*V. cholerae*	P	-
	spicy seafood salad	*V. mimicus*	P	3.9×10^{3}
		V. cholerae	P	5.0×10^{1}
	prawn in fish sauce	*V. cholerae*	P	2.3×10^{3}
	hot and sour soup with prawn	*V. mimicus*	P	-

[a] Presence and absence of *Vibrio* spp. are indicated by P and A, respectively.

[b] The – symbol represents no occurrence by means of surface-spread plate count method, i.e. a level under limit of detection (50 cfu/g).

Since contamination of vibrios in seafood is a problem worldwide (as reviewed above) and the same is assumed for processed and ready-to-eat seafood products, the results of this study are therefore believed to be implicative also for geographical areas other than Thailand. The occurrence or level of the potentially pathogenic *Vibrio* species presented here indicates risks in consumption of undercooked or re-contaminated processed and ready-to-eat (including uncooked) seafood and reaffirms the need to enhance their safety quality. The safety of industrially processed seafood and ready-to-eat seafood

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available at markets/supermarkets or food catering establishments can be of great significance for public health [32,42]. Preventive measures such as proper handling and storage of raw seafood, effective reduction of *Vibrio* spp. in seafood used as raw material, and strict control of safety quality along food processing and food preparation processes (especially in the food industry and food catering unit) should be urgently applied. To promote the safety of ready-to-eat food, of which quality monitoring cannot be carried out routinely, educating food service personnel seems to be the most promising solution.

Table 3. Frequency of positive samples with *Vibrio* spp. revealed by presence-absence and plate count analyses

Seafood category (no. of samples examined)	Number (percentage frequency) of contaminated samples by presence-absence analysis						Number (percentage frequency) of contaminated samples by plate count analysis					
	All vibrios[a]	Specific *Vibrio* species[b]					All vibrios[a]	Specific *Vibrio* species[b]				
		Va	Vc	Vm	Vp	Vv		Va	Vc	Vm	Vp	Vv
industrially processed seafood (16)	4 (25.0)	0 (0)	2 (12.5)	2 (12.5)	1 (6.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ready-to-eat seafood (63)	11 (17.5)	2 (3.2)	7 (10.8)	6 (9.2)	0 (0)	0 (0)	4 (6.3)	1 (1.6)	3 (4.8)	2 (3.2)	0 (0)	0 (0)

[a] all of the 5 species examined.

[b] Va = *V. alginolyticus*, Vc = *V. cholerae* , Vm = *V. mimicus* , Vp = *V. parahaemolyticus* , Vv = *V. vulnificus*.

Conclusions

This study has demonstrated the hazard potential of raw seafood, industrial-scale processed seafood products, and ready-to-eat seafood dishes prepared by food service establishments in relation to potentially pathogenic *Vibrio* spp., which calls for attention and preventive action of the food processing industry, food catering industry, and food authority. It also raises a critical issue in food analysis and standard compliance, as significant differences in the ability of qualitative and quantitative methods to recover *Vibrio* spp. in seafood samples have been demonstrated. The level of *Vibrio* spp. in seafood given here should also be useful for risk assessment concerning pathogenic vibrios.

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