Occurrence of potentially pathogenic bacteria in commercially sold seafood from Misamis Occidental, Philippines

ABSTRACT

Seafood is a nutritious food that establishes one of the enviable components of a healthy diet. Nevertheless, there is health risks linked with the consumption of seafood.
This study aimed to investigate the occurrence of potentially pathogenic *Vibrio* spp., *Staphylococcus aureus*, and *Salmonella* sp. in 200 seafood samples purchased from several retailed outlet in Ozamiz City, Philippines. Different samples analyzed using standard microbiological techniques were the clam, shrimp, squid and fish (anchovy and oil sardines). The *Vibrio* species identified and isolated were *V. parahaemolyticus* (47%), *V. cholerae* (16%) and *V. alginolyticus* (9%). Thirty percent (30%) of the samples analyzed were positive for *S. aureus* and 9% of the samples were contaminated with *Salmonella* sp. The total aerobic plate count mean $\log_{10}$cfu/g of clams, shrimps, head/gills, intestine of fishes (oil sardine, anchovy), and squids were considered as medium to high risk of transmitting pathogenic bacteria. Moreover, the clams and the head/gills of oil sardines were considered as medium to high risk of transmitting pathogenic *V. parahaemolyticus*. The findings revealed that seafood samples analyzed microbiologically were loaded with potentially pathogenic bacteria. This result is of public health concern as these organisms are documented as causes of food-borne diseases.

Keywords:clam, fish, food-born pathogen, shrimp, squid

1. INTRODUCTION

Seafood is vital food component for a large section of world populace [1]. However, there is health risks linked to the ingestion of seafood. One of the main risks involves the consumption of improperly cooked or raw seafood that might be contaminated by foodborne pathogens present in the marine environment [2]. Various outbreaks of bacterial disease associated with the consumption of seafood have been reported for both developed and developingcountries [3]. From these seafood-borne bacteria, *Vibrio* spp. *S. aureus*, and *Salmonella*sp comprise an important group of pathogenic bacteria that frequently causes human disease and even death when the contaminated food is consumed raw or improperly cooked [4].

In the Asian region, *Vibriospp*. has been recognized as the leading cause of foodborne outbreaks in various countries including Japan, India, China, Korea, and Malaysia [5] [6]. Investigations show that many outbreaks may lead to acute gastroenteritis including diarrhea, headache, vomiting, nausea and fever and were caused by ingestion of infected seafood [7]. For food samples, temperature abuse may be instigated by improper storage or a long holding time on the exhibit rack at the retail level without proper temperature control [8] [9]. Among more than 20 Vibrio species identified to be linked with human disease, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are the pathogenic species of *Vibrio* that pose the greatest threat to human health [10]. Most of these *Vibrio* species secrete enterotoxins in water, foods, or in the gastrointestinal tract [11].

Aside for *Vibriospp.* several bacteria in seafood may cause infection to humans. Examples of these bacteria that may cause foodborne illness are *Salmonella*, *Staphylococcus, Shigella,* and *Listeria* [12]. *S. aureus* is one of the most popular sources of gastroenteritis worldwide, which is due to the ingestion of food that has preformed toxins. Humans are

common carriers of *S. aureus* in the nose, throat, and skin infections. Therefore, the organism can be just transmitted to seafood during handling [13].

Seafood is not the usual habitat of *Salmonella*, but still, the occurrence of *Salmonella* in seafood is escalating. Hence, fishery products have been recognized as a major spreader of foodborne pathogens [14][15]. *Salmonella* is associated with fish and fishery products, thereby causing a public health problem. Various authors have reported the incidence of enterotoxigenic *S. aureus* and *Salmonella* [16] in seafood. Infection with these bacteria may affect only the intestines or may spread from the intestinal tract to the blood vessels and then to other body sites.

Though several technological alternatives that could augment the quality of the food supply have been practiced, yet, instances of food-borne diseases caused by consumption of seafood still emerge. In Iligan City, Philippines [17] an outbreak of cholera had killed two people. Health authorities have begun a series of test of water samples in the villages. Other reports said some of those infected individuals were associated with the consumption of improperly cooked seafoods, particularly oyster.
In the provinces of Zamboanga del Sur, Lanao del Norte, and Misamis Occidental Philippines, massive mortalities were observed among pond-cultured shrimp due to *Vibrio harveyi* that was isolated purely from the lymphoid organs and hepatopancreas of affected shrimp and histopathological observations indicated a severe bacterial infection [18]. Disease problems have recorded in shrimp-finfish polyculture in Misamis Occidental due to bacteria [19]. Nevertheless, studies on the presence of potentially pathogenic bacteria on seafoods are scarce. This study aimed to examine the occurrence of potentially pathogenic *Vibrio* sp*., S. aureus*, and *Salmonella*sp in fresh seafood sold from several retailed outlets in the province of Misamis Occidental, Philippines and to highlight that consumption of raw and ingestion of improperly cooked sea foods may pose serious health hazards

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1 Sample collection and processing

Two hundred (200) samples of commercially sold seafood such as clams (40), shrimps (40), squids (20) and fish [anchovy (50) and oil sardines (50)] were purchased from several retailed outlets in the province of Misamis Occidental, Philippines. Samples were placed in sterile, labeled, sealed plastic bags and were transported to the laboratory under the refrigerated condition for analysis. The samples were aseptically removed from the container. After the skin ranging, a sterile forceps and knife appropriate for opening the shell to remove the intestine and gills were used. For shrimp samples, the shell was peeled and detached from the freshly part and intestine. For fishes, samples of head/gills, intestines, and flesh were analyzed microbiologically while only the flesh of squid was examined using standard microbiological techniques. For the clams, the flesh and intervalve water were used for bacterial analyses. About 1g from each sample was weighed and dispensed into 9ml of sterile distilled water and will be shaken vigorously to mix properly. Ten-fold dilutions were made, and dilutions (10^{-6} and 10^{-7}) were selected for microbial enumeration using the standard pour plate method by Fawole and Oso (2001)[20].

2.2 Enrichment procedures

Five (5) grams of each shellfish flesh were incised using a sterile scalpel after removal of the carapace. These 5 g meat samples were homogenized in 45 ml of 3% NaCl containing 1% alkaline peptone water (APW, pH: 8.6) with the use of a sterile blender. The shellfish homogenates were incubated at 37°C for 18 h [21].

2.3 Isolation procedures and identification of bacterial colonies

Analysis of *Vibriospp*. in seafood samples was performed in triplicates. Standard procedures of U.S. Food and Drug Administration (FDA) were used to detect the contamination of potentially pathogenic *Vibrio* species [22] with some modifications. Following incubation, the shellfish homogenates were inoculated using an inoculating loop on Thiosulfate Citrate Bile salts sucrose agar media (TCBS, Hi-Media, India) and incubated at 37°C for 18 h [23]. For each seafood sample, 25g were homogenized in 225ml sterile 0.1% peptone water using a sterile blender. A 10 fold serial dilution was prepared in sterile 0.1% peptone water as described in the Bacteriological Analytical Manual [24]. Spread plate method was carried out using 10 and ten dilutions on TCBS agar. The TCBS plates were incubated at 37°C for 18-24 h and counts were made for each colony type. Discrete colonies were aseptically sub-cultured into fresh agar plates to get pure cultures of colonies.
Colonies recognized as discrete were carefully examined macroscopically for cultural characteristics. Pure isolated colonies were subjected to Gram staining and growing at different salt concentrations by putting the colonies into tubes which contain peptone water and 0%, 3%, 6% and 10% NaCl, and these tubes were incubated at 37°C for 24 h [25]. Also, all bacterial colonies from different samples; growing on TCBS plates were chosen to be streaked on the surface of Trypticase Soya Agar slants (TSA; Oxoid, UK) added with 2% NaCl and incubated for 24 hours at 37°C. Further

identification of Vibrio sp. was carried out using physiological, morphological and different biochemical tests available in the laboratory. Identification of the isolates was performed by comparing their characteristics with those of recognized taxa, as illustrated and described by [26] [27] [28] [29]. To isolate and identify *S. aureus*, 10 g of each seafood samples were homogenized with 90 ml of a sterile 0.1% peptone water solution. Serial dilutions of the homogenate were performed with sterile peptone water and plated in triplicates on Baird Parker agar (Merck 1.05406). After incubation at 35 ºC for 48 h, the distinctive black with apparent halo colonies of *S. aureus* were subjected to Gram staining procedure to clarify bacterial morphology. Staphylococci are spherical Gram-positive bacteria that occur in microscopic clusters). *S. aureus* forms a relatively large yellow colony on rich medium while *S. epidermidis* has a comparatively small white colony [30].

For the detection of *Salmonella* sp., EN ISO 6579 reference method was used. This process currently involves non-selective pre-enrichment in buffered peptone water followed by selective enrichment in Rappaport-Vassiliadis Soya Broth and Muller-Kauffmann tetrathionate/novobiocin broth followed by selective plating of these on Salmonella-Shigella agar. Incubation was made at 35°C, and examine after 24-48 h for colonies resembling *Salmonella sp*. or *Shigella sp*. *Salmonella* will produce hydrogen sulfide (H_2S) gas but will not ferment lactose. The resulting bacterial colonies will appear colorless with black centers. Any suspect salmonella strains that are isolated then confirmed by biochemical and serological test methods.

2.4 Bacterial counts and data analysis

Following proper incubation, dilutions with 30–300 colonies were selected and counted. The number of colony-forming units per g (cfu/g) of seafood was computed by multiplying the number of bacteria by the dilution. Total aerobic plate count (TAPC), *Vibrio* sp., *S. aureus*, and *Salmonella*sp counts that were attained for cfu/g of food were converted into $\log_{10}$ values. Seafood was classified as having a no-to-low risk of transmitting pathogenic bacteria if the TAPC was lesser than 5.0 $\log_{10}$cfu/g and if the counts of Vibrio spp., S. aureus, and Salmonella sp. were less than 3.0 $\log_{10}$cfu/g. Furthermore, if the value is at least 5.0 $\log_{10}$cfu/g for the total bacterial counts and at least 3.0 $\log_{10}$cfu/g for *Vibrio* sp., *S. aureus*, and *Salmonella* sp., the seafood is classified as medium-to-high risk of transmitting pathogen [31][32].

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3. RESULTS AND DISCUSSION

3.1 The Occurrence of *Vibrio spp.*

Seafood samples analyzed microbiologically in this study showed varying degree of bacterial contamination. The results on the contamination of *Vibrio* spp. are summarized in Table 1a & 1b. Of the 200 seafood samples tested, 65.5% were contaminated with potentially pathogenic *Vibrio* spp. The high incidence of *Vibrio* spp. could be due to the nature of these bacteria which is known as halophilic water-borne that commonly dwells in environmental water source worldwide. It has been found that brackish water, as well as marine environments and freshwater, may support the growth of these organisms which are pathogenic to humans [33].

Table 1a Distribution and frequency of occurrence of *Vibrio* spp., isolates from fresh seafood samples.

Samples	No. Tested (%)	No. Positive for Vibrio spp
FISH		
Anchovy	50 (25)	39 (78)
Sardines	50 (25)	42 (84)
SHRIMP	40 (20)	18 (45)
CLAM	40 (20)	22 (55)
SQUID	20 (10)	10 (50)
Total	200 (100)	131 (65.5)

Table 1b. Distribution and frequency of occurrence of *Vibrio* spp., isolates from fresh seafood samples.

Seafood Samples	Number (%) of positive samples

	V. parahaemolyticus	*V. chloreae*	*V. alginolyticus*	Total Positive for *Vibrios* (%)
FISH				
Anchovy				
Gills	14 (28)	06 (12)	04 (08)	24 (48)
İntestine	05 (16)	04 (08)	02 (04)	11 (22)
Meat	04 (08)	00 (00)	00 (00)	04 (08)
SARDINES				
Gills	16 (40)	04 (08)	02 (04)	22 (44)
İntestine	8 (20)	02 (04)	02 (04)	12 (24)
Meat	06 (12)	02 (04)	00 (00)	08(16)
SHRIMP	10 (25)	06 (15)	02 (05)	18(45)
CLAM	12 (30)	06(15)	04(10)	22(55)
SQUID	06 (30)	02 (10)	02 (10)	10(50)
Total number (%) of positive samples	81 (40.5) /200	32 (16)/200	18 (09) /200	131(65.5)

The samples that harbored most of the *Vibrio* spp. were the fish in which the gills obtained most of the isolates. This could be due to the gills physiology which serves as a sieve that filters water that comes into the fish from their water ecosystem. Fish could be predisposed to gill disease because gill is used for the exchange of gasses and the fish will be hassled because of intricacy in the exchange due to heavy load of microbes in water then the gill is to be infected [34].

Among the *Vibrio* species isolated, *V. parahaemolyticus* was the predominant (40.5%) followed by *V. cholera* (16%) and *V. alginolyticus* (09%). The findings of this study agree favorably with the results of several authors in a similar study conducted in Brazil, China, India and Bursa province, Turkey [35][36]. On the other hand, varying rates were reported in some other studies. In Nigeria, *V. chloreae* was the most frequently isolated *Vibrio* sp. followed by *V. parahaemolyticus*[37].

The predominant *V. parahaemolyticus* is a salt tolerant but very sensitive to heat and is destroyed by cooking. However, this is a common cause of foodborne diseases in many Asian countries such as China, Japan, and Taiwan, and is recognized in the United States as the leading cause of human gastroenteritis [38]. This species is linked with the consumption of insufficiently or raw cooked seafood, poor handling of seafood during preparation and improper post-harvest storage conditions [39].This pathogen is commonly isolated from a variety of seafood including oyster, crab, scallop, lobster, shrimp and fish [40].

In the present study, 16% of the samples tested showed positive of *V. cholerae*. This bacterium favors alkaline surroundings to thrive well [41]. Perhaps the presence of this bacterium was linked to the low salinity in various locations due to the influx of freshwater to the ocean. This species can cause cholera and is typically a waterborne illness. Food-borne cases primarily fish outbreaks are also known carrier of this microorganism.

In this study, the prevalence of *V. alginolyticus* in seafood was 9%. This finding was nearly close to the result of Baffone et al (2006), who reported 8.16% in clams in Italy. In contrast, Hassanin (2007) cited higher infection rates for *V. alginolyticus* in shrimps in Egypt. This bacterium is one of the most popular *Vibrio* species occurring in the marine environments and seafood (Neetoo et al 2022). *V. alginolyticus* is an opportunistic pathogen, and its pathogenicity is thought to be similar to that of *V. parahaemolyticus*[42]. In India and Taiwan, this *Vibrio* species is linked with a white spot in shrimp while the zoonotic risk of this pathogen has been implicated in soft tissue, wound and ear infections in human [43]. *Vibrio* spp. and several pathogenic species are known to be commonly associated with outbreaks of *Vibrio* infections due to ingestion of raw or improperly cooked seafood, water contaminated with human feces or sewage, inappropriate post-harvest storage conditions or poor handling of seafood during preparation.

3.2 The occurrence of S. aureus and Salmonella sp.

The incidence of *S. aureus* in this study was found to be higher than *Salmonell*a sp. *S. aureus* was detected in 30% of the 200 raw samples of seafood, whereas the incidence of *Salmonella*sp was 9%. The head/gills of fishes (oil sardines & anchovy) were heavily loaded with *S. aureus*. Four isolates of *S. aureus* were obtained from shrimp samples, three isolates from clams and two isolates from squids (Table 2).

It is well documented that freshly caught seafood are free from *S. aureus*, and contamination takes place upon handling [44]. Other conditions, such as poor personal hygiene, inadequate refrigeration, and delay in processing are associated with staphylococcal contamination. *S. aureus* is a major cause of food poisoning as well as of a range extraintestinal infection [45]. Staphylococcal food poisoning is caused by a preformed enterotoxin produced by growth of the bacteria in food that is ingested [46].

The overall prevalence of *Salmonella* sp. in 100 seafood samples tested was in agreement to that reported elsewhere. The findings of this present study showed that the organism was not recovered from the gills and flesh of the fish, but was isolated from their intestine only. This result is in agreement with the work of Green [47]in which the organism was never

recorded from the flesh of the fish, but was documented from viscera and epithelium. Several scientists believe that fish are possible carriers of *Salmonella* which are accumulated in their intestines for somewhat short periods of time and some believe that fish get actively infected by *Salmonella*[48][49]. Furthermore, the mollusk and the crustacean samples also harbored colonies of *Salmonella*.

The possible cause of *Salmonella* contamination in seafood might be due to farm runoff and fecal contamination from nearby domesticated or wild animals and poor water quality. In addition to poor distribution, retail marketing, handling, and high stocking, preparation practices, densities and high water temperature may be responsible for increased contamination of Salmonella in shrimp [50](Loest et al 2022). Other investigations concerning live bivalve mollusks confirmed that this bacterium could endure coastal water salinity. The bacteria may found in 0%-2.5% of live shellfish and intake of these foods may cause salmonellosis, characterized by enteric fever along with gastroenteritis and diarrhea. Similarly, the U.S. Food and Drug Administration's (FDA) data revealed that the most common contaminant of fish and fishery products was *Salmonella*[51]. About 12% of the foodborne outbreaks related to consumption of fish are due to bacteria including *Salmonella*.

Table 2. Distribution and frequency of occurrence of *Salmonella sp*. and *S. aureus* isolates from fresh seafood samples.

Seafood Samples	Number (%) of positive samples / number of samples tested	
	Salmonella sp.	*S. aureus*
FISH		
Anchovy		
Gills	00 (00) /50	08 (16)/50
İntestine	02 (04) /50	06 (12)/50
Meat	00 (00) /50	04 (08)/50
Sardines		
Gills	00 (00) /50	12(24)/50
İntestine	04 (08) /50	04/(08)50
Meat	00 (00) /50	08(16)/50
SHRIMP	04 (10) /40	06(15)/40
CLAM	06(15)/40	08(20)/40
SQUID	02 (10) /20	04(20)/20
Total number (%) of positive samples/Total number of samples tested	18 (09) /200	60 (30) /200

3.3Bacterial Counts in Seafood

The mean $\log_{10}$cfu/g of *Vibrio* spp., *S. aureus,Salmonella* sp. and total aerobic plate counts (TAPC) values were presented in Table 3. The sample of clams and shrimps tested obtained the highest TAPC value of 7.61 and 7.48 respectively. These molluscan bivalves and decapods crustaceans are filter feeders, and they may be apt to hoard microbes in the nearby waters which may also contain these microorganisms. They are typically grown-up and collected in near-shore; shallow estuarine waters [52] and are therefore expected to harbor high concentrations of pathogenic organisms. If this seafood is eaten after a very mild heat treatment or raw, they pose a significant health risk to the consumers. The seafood which may be linked with human infection caused by *V. parahaemolyticus* are bivalve mollusks, crustacean and finfish especially those served raw or superficially cooked and which have been exposed to temperature abuse[53]. Of the fishes, the head/gills of oil sardines and anchovy harbored considerable TAPC value of 7.42 and 6.78.

The TAPC value suggests that samples of shrimps, clams, head/gills and intestines of fishes as well as squids examined in the study were considered as a medium to high risk of transmitting pathogenic bacteria (not within acceptable limits, i.e. <5.0 log10 cfu/g for total counts). Moreover, the clams and the head/gills of oil sardines were also considered as medium to high risk of transmitting pathogenic *V. parahaemolyticus*. From the zoonotic point of view, contamination of potentially

pathogenic bacteria in all samples of seafood tested suggests a possible risk to the health of people consuming raw or improperly cooked seafood. Naturally fish and other seafood have immunity for infections, but their immunity decreases when the concentration of the microorganisms is on the increase [54]. Bacteria in seafood improperly cooked could be transmitted to man as they establish themselves in the intestine particularly those potentially pathogenic causing problems including diseases of various kinds. These bacteria enter into the body and then discharge endotoxins which irritate the stomach and the bowels. This toxin might lead to stomach cramps diarrhea and fever and is called foodborne infection.

Table 3 Total aerobic plate counts, *Vibrio*spp, *S. aureus* and *Salmonella* spp counts mean $\log_{10}$cfu/g (ml) ± standard deviation of Seafoods in Ozamiz City.

Seafood Item	n	TAPC	*Vibrio* isolates			*S. aureus*	*Salmonella*
			V. parahaemolyticus	*V. alginolyticus*	*V. chloreae*		
Fish							
Anchovy	25						
head/gills		6.78±1.7	2.87±1.0		1.23±0.7	1.02±0.6	1.19±0.4	00
intestine		5.60±0.5	1.14±0.9		1.70±0.9	0.37±0.6	1.10±0.5	1.66±1.2
meat		3.64±2.0	1.05±1.1		00	00	1.20±0.1	00
Oil Sardines	25						
head/gills		7.42±2.1	3.04±0.4		1.21±1.0	1.03±0.1	2.19±0.5	00
intestine		5.90±1.9	1.06±0.5		1.07±1.6	1.87±0.6	1.19±0.5	1.15±0.3
meat		3.51±1.0	1.04±0.7		1.02±0.8	00	1.05±0.5	00
Shrimp	20	7.48±1.8	2.69±0.7		1.10±0.7	0.87±0.8	1.73±1.1	1.06±1.8
Clam	20	7.61±1.5	3.14±0.5		1.05±0.4	0.89±0.4	1.93±0.5	1.59±0.2
Squid	10	5.55±0.9	1.20±0.2		1.02±0.2	1.04±0.9	1.14±1.3	1.01±0.9

4. CONCLUSION

Seafood is considered as an essential element in the diet and plays a vital role in the appearance of foodborne diseases. This study revealed that seafood samples that were analyzed microbiologically could be a source of foodborne bacterial pathogens such as *Vibrio sp. S. aureus and Salmonella*. The contamination of these bacteria could be attributed to cross-contamination from surroundings, handling by the sellers and thus, constitute a potential health hazard to the public.

Nevertheless, fishes and other seafood have to be properly cooked before consumption, and proper quality control measures have to be adopted in culturing, processing, harvesting and consumption of seafood. Therefore, monitoring of the contamination of potentially pathogenic bacteria in harvested seafood is vital for sustenance of public health. Further studies have to be conducted to include other seafood products in the microbial analyses and examine other pathogens probably present in seafood.

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