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REVIEW

Vibrio parahaemolyticus and *Vibrio cholerae*

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Abstract

This review aimed to provide insights on *Vibrio parahaemolyticus* and *Vibrio cholerae*. The review focused on the current scenario of *V. parahaemolyticus* and *V. cholerae* in Malaysia, strains characterisation and their foodborne outbreaks. The isolation and identification procedures will be reviewed as well to discuss emerging detection methods. The risk factors of *V. parahaemolyticus* and *V. cholerae* were highlighted in this review to prevent future foodborne outbreaks which can affect the political, social and economy of the country.

1. Introduction

Malaysia, a tropical country, strategically located in the middle of Southeast Asia is blessed with several marine aquacultures such as seafood, fish and other fishery products. Malaysia is an important producer, market and trading nation for fish and fishery products in the region. The Malaysia society consisting of multiple-ethnic has recorded a high per capita seafood consumption of more than 50 kg/year, one of the highest in Asia, ranking second behind Japan (INFOFISH, 2012). The main driving factors for the increased demand of seafood is mainly due to changing consumer lifestyle towards healthy food and convenience products, and the growing tourism and hospitality industries. Although consumption of fresh seafood especially fish is deemed healthy, we are not free from threat of foodborne illness. This is mainly because of all year long hot and humid climate in Malaysia provides a very suitable condition that supports the growth of microorganism especially *Vibrio* spp. in the estuarine environment.

Vibrio spp. are Gram-negative, motile curved rod-shaped bacteria with a single polar flagellum. Vibrios are the predominant genus of bacteria which has been greatly associated with seafood. *Vibrio* spp. may possibly be isolated in approximately 40-60% of finfish and shellfish sold at retail market (Montville *et al*., 2012). This disease-causing species of pathogens occur naturally in marine, coastal and estuarine (brackish) environments and are most abundant in estuaries of this region. Over 20 *Vibrio* species have been described with at least 12 being capable of causing illness in human. Among these pathogenic species, eight species can cause or associated with foodborne illness (FAO/WHO, 2002).

Vibrio spp. have been recognised as one of the leading causes of foodborne outbreaks in many Asian countries including Japan, India, China, Taiwan, Korea and Malaysia (Noorlis, Ghazali, Cheah, Tuan Zainazor, Ponniah *et al*., 2011). Among the *Vibrio* species, *V. cholerae* and *V. parahaemolyticus* are known as the most potential and emerging water-borne pathogens responsible for negative impact to humans, marine animals and aquaculture. In Malaysia, food intoxication related to *V. cholerae* poses a public health implication as sporadic outbreaks that occur periodically. Meanwhile, *V. parahaemolyticus* has been identified as the most prominent cause of human gastroenteritis associated with seafood consumption in Asia (Vimala *et al*., 2010). This will eventually cause serious impact on the social-economic issues in the affected population.

Such impact prompted the demand for effective and efficient detection/identification of *Vibrio* spp. in the food supply chain. The traditional method of isolation

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and enumeration of *Vibrio* spp. involved the most probable method (MPN) coupled with traditional confirmation techniques. This method has several drawbacks due to its laborious and time-consuming nature (Nishibuchi, 2006). The development of molecular methods involving polymerase chain reaction (PCR) for detection of pathogens has proven to provide quantitative and qualitative determination of the organism (Cockerill, 2003). MPN coupled PCR was reported to be more efficient and effective with significant time saving and labour saving (New *et al*., 2014). Such detection method has enabled the researcher to efficiently investigate or detect the presence of *Vibrio* spp. and allow relevant authorities to take appropriate precaution measures.

This chapter provide an overview of the pathogenic strains of *Vibrio* spp. specifically *V. cholerae* and *V. parahaemolyticus*. The scenario of these bacteria in local population including their prevalence in food and environment, characteristics of the pathogenic strains, incidences of outbreak, case studies and detection method are discussed. This chapter also describes the social-economic impact of *Vibrio* spp. and its control and preventive measures.

2. Current scenario of *Vibrio parahaemolyticus* and *Vibrio cholerae* in Malaysia

2.1 Prevalence

Vibrio spp. are natural inhabitants of freshwater, estuarine and seawater environments. Therefore, aquatic environment acts as a reservoir and source of transmission. In temperate and tropical regions such as Malaysia, these bacteria are ubiquitous in aquatic environments and are abundant in estuaries throughout the year (Vimala *et al*., 2010). Previous study has shown that pathogenic *V. parahaemolyticus* were found in water and sediment of Matang mangrove estuaries which could pose a high health risk to the public (Ghaderpour *et al*., 2014). Potentially pathogenic *V. parahaemolyticus* were also reported as frequent contaminants in coastal seawater of west region of Peninsular Malaysia (Morib, Kuala Lukut and Port Klang).

In addition to the aquatic environment and estuarine of Peninsular Malaysia, *V. parahaemolyticus* were also found to be present in aquatic environment of eastern Malaysia. In west coast of Sabah, Malaysia, 11 species of *Vibrio* were reported to occur with varying percentage in 72 environmental samples collected from six aquaculture sites. Among the species, *V. parahaemolyticus* (22.2%) was shown to exhibit highest prevalence in the aquaculture waters of the west coast of Sabah, Malaysia (Ransangan *et al*., 2013). *Vibrio parahaemolyticus* were also reported to present consistently over twelve weeks (100% presence of *V. parahaemolyticus*) in 48 water samples collected from 2 sites of estuarine region of Sarawak River which is nearer to seawater region (Lesley *et al*., 2014). However, the occurrence of *V. parahaemolyticus* in estuarine region nearer to freshwater site were relatively lower compared to the region nearer to seawater site where the freshwater site showed only 17% presence of *V. parahaemolyticus* in the water sample. These were mainly due to the salinity of the estuarine site. The abundance of *V. parahaemolyticus* counts was positively correlated with the water salinity due to the fact that *V. parahaemolyticus* is a halophile.

Such contamination in the aquatic environment and estuarine may lead to contamination of fishery and aquaculture products such as prawns, fishes and blood cockles. The occurrence of pathogenic *Vibrio* in seafood was supported by Vengadesh *et al*. (2012) who reported the presence of *V. cholerae* in all (100%) samples collected from wet market and 22.8% of samples from supermarket. The occurrence of *V. cholerae* in various seafood samples from the market is probably a reflection of the atmosphere at the supermarket and wet market. The contamination could occur due to the way of handling seafood at wet markets, which is less hygienic as compared to supermarkets (Vengadesh *et al*., 2012). In addition to *V. cholerae*, *V. parahaemolyticus* was also found in seafood samples (shrimp) as analysed with conventional plating method. The mean total *Vibrio* count in the shrimp samples ranged from 4.36 log CFU/mL to 6.34 log CFU/mL (Letchumanan *et al*., 2015). Several other pathogenic *Vibrio* species were also isolated from various seafood samples obtained in seafood markets and supermarkets at 11 sites selected from four states in Malaysia between July 1998 and June 1999 (Elhadi *et al*., 2004). The authors examined 768 sample sets including shrimp, squid, crab, cockles, and mussels and found that eight potentially pathogenic *Vibrio* spp. were detected. The overall incidences in the samples were 4.6% for *V. cholerae*, 4.7% for *V. parahaemolyticus*, 6.0% for *V. vulnificus*, 11% for *V. alginolyticus*, 9.9% for *V. metschnikovii*, 1.3% for *V. mimicus*, 13% for *V. damsela*, 7.6% for *V. fluvialis*, and 52% for a combined population of all of the above. The high incidence of *Vibrio* species in seafood could be due to ubiquitous presence of this pathogen in estuarine environments and coastal waters.

Among the seafood samples, cockles (*Anadara granosa*) showed the highest percentage of incidences (82%) and the incidences were highest in Kuching, Sarawak with 100% of incidence (Elhadi *et al*., 2004). Elsewhere, Bilung *et al*. (2005) also demonstrated high

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incidence of *V. parahaemolyticus* is cockle samples (62%) obtained from Tanjong Karang, Kuala Selangor, Malaysia. In another study, Suzita *et al*. (2010) also reported the potential risk of illness associated with the consumption of cockles harvested from east and west coast of Malaysia. It was reported that 24% of the cockle sample possess high density of *V. cholerae* (> 24000 MPN/g). All these suggested that consumption of cockles may pose a health hazard to the consumers especially when the contaminated food is undercook or store at ambient temperature. Storage at such condition for few hours will lead to further contamination that may possibly reach the infectious dose.

In addition, the prevalence of *Vibrio* spp. and *V. parahaemolyticus* was also found to be high in freshwater fish, *Pangasius hypophthalmus* (catfish) and *Oreochromis* spp. (red tilapia) sold in hypermarket at Selangor, Malaysia. The prevalence of *Vibrio* spp. and *V. parahaemolyticus* in these samples (150 samples) which was collected over a 5 month period was reported to be 99% and 24%, respectively with higher percentages detected in samples from the gills followed by the intestinal tract and flesh (Noorlis, Ghazali, Cheah, Tuan Zainazor, Ponniah *et al*., 2011). Anuar (2012) also reported the presence of *V. cholerae* in various fish species namely, *Mystus* spp., *Arius sagor*, *Punctius gonionotus*, *Oreochromis niloticus*, *Mystus castaneus*, *Diodon nicthemerus* and *Neolissochilus thienemanni* that has been collected from three different tributaries of Sarawak River, Sabang River, Tuang River and Kitang River. This outcome of biosafety assessment of *Vibrio* spp. in freshwater fish indicates another potential source of food safety issues to consumers. Other studies indicating the prevalence of *V. cholerae* and *V. parahaemolyticus* in Malaysian food and environmental samples are presented in Table 1.

Although Vibrios are known to be easily killed by heat, post-cooking contamination may lead to food poisoning as a result of Vibrios persistence in seafood-related products. In a recent study, Tang *et al*. (2014) demonstrated that *V. cholerae* may survive well in Malaysian fish sausage snack, '*keropok lekor*' (boiled and fried) particularly when it is stored in a closed container. The authors demonstrated that *V. cholerae* survived at 7.95±0.05 log CFU/g (boiled) and 6.11±0.18 log CFU/g (fried) in '*keropok lekor*' after storage at closed container for 6 h. Although heat treatment or cooking may significantly reduce the hazard, this study may indirectly raise the safety concern for consumption of cooked food which has been exposed to post-cooking contamination.

Various studies have extensively reported the occurrence of *Vibrio* spp. in water and seafood sample. However, there has been limited study that evaluated the prevalence of these bacteria in vegetables which are commonly eaten raw. Tunung *et al*. (2010) investigated the biosafety of *V. parahaemolyticus* in raw salad vegetables at wet markets and supermarkets in Malaysia and found that the occurrence of *V. parahaemolyticus* was 20.7%, with a higher frequency of *V. parahaemolyticus* in vegetables obtained from wet markets. The contamination of *Vibrio* spp. in vegetable

Table 1. Prevalence of *V. cholerae* and *V. parahaemolyticus* in foods in Malaysia

Species	Sample/Source	Prevalence	References
V. parahaemolyticus	Cockles	27/100 (27.0%)	Son, Nasreldin, Zaiton *et al*. (1998)
V. cholerae	Surface water	4/60 (6.7%)	Son, Rusul, Samuel *et al*. (1998b)
V. cholerae	Street foods	(0.7%)	National Public Health laboratory/ Ministry of Health (NPHL/MOH) (2005a)
V. cholerae	Frozen squids	2/146 (1.4%)	NPHL/MOH (2005b)
V. parahaemolyticus	Frozen prawn	42/455 (9.2%)	
V. parahaemolyticus	Seafood	43/150 (29%)	Paydar *et al*. (2013)
V. parahaemolyticus	Oyster	15/30 (50.0%)	New *et al*. (2014)
V. parahaemolyticus	Water of aquaculture farm	132/264 (50.0%)	Pui *et al*. (2014)
	Shrimp	11/27 (40.7%)	
tdh+ V. parahaemolyticus	Molluscan shellfish (bloody clams and surf clams) and crustaceans (shrimps)	77/232 (33.1%)	Tan *et al*. (2015)
trh+ V. parahaemolyticus		16/232 (6.9%)	
V. parahaemolyticus	Vegetables	3/30 (10.0%)	New *et al*. (2016)
	Seafood	5/15 (33.3%)	
V. parahaemolyticus	Short mackerels (*Rastrelliger brachysoma*)	116/130 (89.2%)	Tan *et al*. (2017)
tdh+ and/or trh+ V. parahaemolyticus		21/130 (16.2%)	

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could possibly be due to the contamination by soil irrigated with contaminated water (Okafo *et al*., 2003). Although there has been limited study to support the prevalence of *Vibrio* spp. in vegetable in Malaysia, this study suggested that raw vegetables may also act as a transmission route for *V. parahaemolyticus*. This may cause a safety concern to consumer as salad vegetable are commonly eaten raw in Malaysia without further heat treatment to reduce or eliminate the bacteria count.

2.2 Surveillance

Surveillance is a timely and organised collection of existing clinical or laboratory data regarding the well-being of a particular population. The public health program in many countries has given high priority to the surveillance of foodborne diseases. The surveillance data is particularly essential for estimating the burden of foodborne diseases and identifying emerging food safety issues, and subsequently evaluating the impact of control measures undertaken. This surveillance system may vary from country to country depending on its infrastructure, economic status, availability of manpower and technical expertise (Sharifa *et al*., 2013).

In Malaysia, such data are obtained from notifications of diseases, laboratory reports, environmental indices (e.g. food establishment inspection sources, agriculture, veterinary and food analyses), outbreak investigation reports, research studies, morbidity reports, case investigations, sentinel reports, surveys, census and media reports (FAO/WHO 2004). The notification is based on the syndrome present from the surveillance of foodborne disease rather than a specific disease. This syndromic notification is beneficial since it facilitates timely cautionary and enables rapid response to disease outbreak without being deferred by the laboratory confirmation. Additionally, the Ministry of Health Malaysia also conducts laboratory-based surveillance of specific infectious diseases including foodborne diseases.

3. Strains characterisation

3.1 Vibrio cholerae

Vibrio cholerae is a Gram-negative motile bacterial species where O1 and O139 strain (isolated from Bengal) was reported to be the main causative agent of cholera (Ramamurthy *et al*., 1993). These strains of *Vibrio* usually produce cholera toxin (CTX), the major factor contributing to food poisoning outbreak that occurs periodically in Malaysia. This indicated that it is important to identify the characteristic of the *Vibrio* strains from water environment and seafood in Malaysia as not all *V. cholerae* will cause illness.

The existence of toxigenic strains in Malaysian water and seafood samples has been reported by several previous studies. Chen *et al*. (2004) isolated 97 strains of *V. cholerae* from various Malaysian seafood samples and reported that 20 strains carried the *ctx* gene and produced cholera toxin. Among the toxigenic strains, 14 belonged to the O139, 1 belonged to O1 Ogawa, and 5 belonged to rough (R) serotypes. The authors also demonstrated that the R strains and O1 strains that were isolated from seafood possess a close relationship, suggesting the possibility that these R strains were derived from an O1 Ogawa strain(s) by mutation in the somatic antigen (Chen *et al*., 2004). In another study, 29 Malaysian strains were tested and 1 was subtyped as O139 serogroup, 7 were non-O1/ non-O139, and 21 were subtyped as O1 serogroup. All these strains harbour toxigenic genes including *toxR*, *ompW*, *hlyA*, *ctxA* and *tcpl*. This was also supported by other studies who demonstrated that *V. cholerae* isolated from a cholera outbreak in Kelantan, Malaysia and Miri, Sarawak, Malaysia were toxigenic, harbouring virulence genes including *ace*, *zot* and *ctxA* (Son *et al*., 2002; Ang *et al*., 2010). All these studies show that many of the strains isolated from Malaysian samples harbour toxigenic genes that may pose danger to public health.

In addition to toxigenic genes, most of the strains isolated from Malaysian samples also possess resistant to multiple antibiotics. Ang *et al*. (2010) demonstrated that 20 isolates from clinical samples were found to be resistant to multiple antibiotics, including tetracycline, erythromycin, sulphamethoxazole/trimethoprim, streptomycin, penicillin G, polymyxin B and ampicillin. The resistance to multiple antibiotics was also observed in strains isolated from freshwater fish in Malaysia. These isolates showed particularly high resistance to different antibiotics including bacitracin, vancomycin, tetracycline, furazolidone, cephalothin and erythromycin (Noorlis, Ghazali, Cheah, Tuan Zainazor, Wong *et al*., 2011). The resistance to furazolidone, bacitracin and vancomycin was observed in 100% of the *V. cholerae* isolates followed by tetracycline (88%), cephalothin (75%) and erythromycin at 63%. The high resistant level of furazolidone, bacitracin and tetracycline were of concern as these antibiotics are commonly the drug choice to treat *Vibrio* infection. These can also be a potential threat in our region as the outbreak caused by these *Vibrio* spp. may not be treated efficiently. Generally, the resistance of isolates (from clinical and freshwater fish samples) towards ciprofloxacin, norfloxacin, chloramphenicol, gentamicin and kanamycin were considerably lower compared to other type of antibiotics (Ang *et al*., 2010; Noorlis, Ghazali, Cheah, Tuan Zainazor, Wong *et al*., 2011).

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3.2 Vibrio parahaemolyticus

Vibrio parahaemolyticus is a Gram-negative halophilic pathogen which is widely responsible for human gastroenteritis worldwide. Cases of *V. parahaemolyticus* were mostly sporadic and associated with diverse serovars. Since 1996, O3:K6 serovar has emerged as the predominant strain causing pandemics in many Asian countries. Fortunately, there has yet to be any reported outbreaks related to *V. parahaemolyticus* in Malaysia (Cheah *et al*., 2013). However, this does not suggest that consumption of Malaysian seafood is safe from *V. parahaemolyticus* infection.

Various studies have reported the presence of virulent *V. parahaemolyticus* strains carrying *tdh* and *trh* gene from coastal seawater and seafood in Peninsular Malaysia. The presence of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) encoded by the genes *tdh* and *trh*, respectively is always considered as markers of pathogenicity in *V. parahaemolyticus*. Cheah *et al*. (2013) reported that *V. parahaemolyticus* isolated from local shellfish such as bloody clam (*Anadara granosa*) and lala (*Orbicularia orbiculata*) carried both *tdh* and *trh* gene. Virulent strains carrying *tdh* and *trh* genes were also found in other seafood such as shrimp and cockles (Al-Othrubi *et al*., 2014). The authors demonstrated that 12.3% of the strains isolated from shrimp and cockles were positive for *tdh* virulence gene (3 isolates from shrimp and 5 isolates from cockles), whereas 40% isolates were positive for *trh* virulence gene (9 from shrimp and 17 from cockles). Similarly, Bilung *et al*. (2005) evaluated the prevalence of *V. parahaemolyticus* in cockles (*Anadara granosa*) obtained from Tanjong Karang, Kuala Selangor (October to November 2003) and the PCR analysis revealed that 2 samples were positive for *tdh* gene and 11 were positive for *trh* gene. The presence of virulent genes in environment and aquatic samples were further strengthened by Sujeewa *et al*. (2009) where they reported that 15% of the isolates from culture environment (from live shrimp, sediments and water) and 7% of frozen shrimp samples were positive for *tdh* and *trh* genes. The occurrence of virulent strains in the environment and seafood samples suggests a probable health risk for people consuming local raw seafood.

In addition to toxigenic gene, development of multiple drug resistance among *V. parahaemolyticus* is another issue of concern. *V. parahaemolyticus* isolated from water of six aquaculture sites (shrimp hatchery and grow-out farm) along the west coast of Sabah, Malaysia were shown to be sensitive to chloramphenicol, nalidixic acid and oxolinic acid but resistant to ampicillin, penicillin and vancomycin (Ransangan *et al*., 2013). In agreement, twenty-one *V. parahaemolyticus* isolates from coastal seawater from three beaches in peninsular Malaysia were found to exhibit multiple antibiotics resistance (MAR) with MAR indices of 0.29 to 0.57 (Tanil *et al*., 2005). MAR index values greater than 0.2 suggest that it is a high-risk source of contamination where antibiotics are often used (Poonia *et al*., 2014). The resistance of the twenty-one *V. parahaemolyticus* isolates was observed toward penicillin (100%), ampicillin (95.2%), carbenicilin (95.2%), erythromycin (95.2%), bacitracin (71.4%), cephalothin (28.6%), moxalactam (28.6%), kanamycin (19.1%), tetracycline (14.3%), nalidixic acid (9.5%) and gentamicin (9.5%) (Tanil *et al*., 2005).

Considering that there is a strong correlation between the water and aquaculture residing in the water, the multiple drug resistance characteristic was also observed in various *V. parahaemolyticus* isolates obtained from aquacultures such as fish, shrimp and others. Noorlis, Ghazali, Cheah, Tuan Zainazor, Ponniah *et al*. (2011) evaluated the antibiotics sensitivity of 49 strains of *V. parahaemolyticus* isolated from freshwater fish of patin (*Pangasius hypopthalmus*) and red tilapia (*Oreochromis* spp.) purchased from different retail levels in Selangor, Malaysia and found that the isolates showed the highest prevalence of resistance towards bacitracin (98%), tetracycline (82%), furazididone (82%), cephalothin (76%), erythromycin (68%) and vancomycin with 65% resistance level. on the other hand, the resistance towards other antibiotics such as amikacin (45%), norfloxacin (39%), chloramphenicol (37%) was lower and the least resistant was toward imipenem with only 12%. In another study, Al-Othrubi *et al*. (2014) also demonstrated that *V. parahaemolyticus* isolated from cockles and shrimp (Selangor, Malaysia) between July 2011 and August 2013 possessed multiple drug resistance with high resistance to common antibiotics including ampicillin (63.1%) and cephalexin (35.4%). In addition, 83% of *V. parahaemolyticus* strains in shrimps purchased from local wet markets and supermarkets also showed multiple antibiotics resistance (MAR) with a MAR index of more than 0.2. As the fish and other aquaculture are used for human consumption, the development of antibiotic resistance in pathogens could present a health risk to the consumer.

4. Foodborne outbreaks

Vibrio cholerae is the cholera-causing culprit worldwide, especially in countries that lack clean water supplies, poor sanitary system and insufficient public health facilities (Mandomando *et al*., 2007). Contaminated drinking water and undercooked seafood are sources of cholera outbreak in Malaysia (Lim 2001).

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Malaysia lies in the cholera endemic Southeast Asia zone and experienced serious cholera outbreaks between 1991 and 1994 (Mahalingam *et al*., 1994). Cholera outbreaks caused by the El Tor O1 *V. cholerae* serogroup occur periodically, and outbreaks caused by the 0139 serogroup occur sporadically (Chen *et al*., 2004; Ang *et al*., 2010; Teh *et al*., 2011). However, the non-O1/ non-O139 *V. cholerae* serogroup has not been reported to cause any serious cholera outbreak.

In 2001, more than 60% (371/557) of the total national cases were reported in Sabah while two separate outbreaks (total 124 cases) occurred in Selangor (Petaling Jaya and Klang districts). In 2002, the occurrence of a small cholera outbreak in Penang was caused by the 0139 serogroup. In the same year, a big cholera outbreak occurred in Selangor among the students of a semi-boarding school. In 2004, a total of 16 confirmed cases and 22 carriers were identified in Kota Setar district (Kedah) while 27 confirmed cases and four carriers were from Baling district (Kedah). A total of thirty O1 *V. cholerae* and two O139 *V. cholerae* isolates were collected from infected patients in Malaysia between 1999 and 2003 for genotypic characterisation (Patrick *et al*., 2012). Patrick *et al*. (2012) revealed that all collected isolates were divided into two main groups, which were subdivided into two clusters each based on their geographical location. All these isolates have been proven to contain *ctx* gene by PCR and exhibited low resistance to all the tested antibiotics (except the 0139 serotype) (Patrick *et al*., 2012).

The latest outbreaks of cholera were reported in East Coast Islands, Kelantan and Terengganu (Ang *et al*., 2010; Teh *et al*., 2012). In November 2009, a cholera outbreak occurred in Terengganu was caused by two El Tor *V. cholerae* variants (Teh *et al*., 2012). The outbreak spread from Kuala Terengganu to several districts within seven days. This outbreak was then controlled in late November with approximately 400 persons hospitalised as well as 187 cases and 1 death were confirmed (Teh *et al*., 2012). This study collected the rectal swab samples from patients admitted to Hospital Sultanah Nur Zahirah in Kuala Terengganu who had acute diarrhoea during the outbreak period. A total of 37 isolates from the rectal swab samples were confirmed as *V. cholera* El Tor O1. These clinical O1 *V. cholerae* isolates were found to be resistant to ampicillin, trimethoprim/sulfamethoxazole, erythromycin and tetracycline. Generally, tetracycline has been considered the drug of choice for cholera treatment, but it has been replaced by erythromycin due to increase number of tetracycline-resistant strains since 1992. The emergence of multi-resistant isolates in this 2009 outbreak may suggest that there is a need of alteration for clinical management of cholera in Malaysia.

As with neighbour to Terengganu, another cholera outbreak occurred in Kelantan (six different districts) between November and December 2009 (Ang *et al*., 2010). A total of 33 cholera cases were confirmed within three weeks, which was caused by a single clone of El Tor *V. cholerae* strain. Most of the patients (21/33, 64%) are local Kelantanese and the remaining 12 were migrant Thai workers. All these isolates were resistant to multiple drugs, including tetracycline, erythromycin, sulfamethoxazole-trimethoprim, streptomycin, penicillin G and polymyxin B.

5. Isolation and identification of *Vibrio cholerae* and *Vibrio parahaemolyticus*

5.1 Enrichment broth and selective plating media

Diagnoses of infections caused by *V. cholerae* and *V. parahaemolyticus* have conventionally relied on the isolation of the pathogens from cultures of stool, wound, or blood. Various different enrichment broths, such as salt polymyxin broth (SPB), alternative protein source (APS) broth, salt colistin broth, glucose salt teepol broth, and bile salt sodium taurocholate (ST broth) were used for the isolation of *Vibrio* pathogens, although alkaline peptone water remains the mostly used and recommended by the U.S. Food and Drug Administration (FDA) as the enrichment broth for all *Vibrio* species (DePaola and Kaysner 2004). *Vibrio* species grow more rapidly in alkaline peptone water (6 to 8 hrs) than non-*Vibrio* microorganisms. Raghunath *et al*. (2009) isolated more pathogenic *V. parahaemolyticus* strains from seafood samples by using ST broth than alkaline peptone water.

Selective medium containing thiosulfate, citrate, bile salts, and sucrose (TCBS agar) is recommended to isolate not only for *V. cholerae* and *V. parahaemolyticus* but all other pathogenic *Vibrio* spp. except for *Vibrio hollisae* (Kobayashi *et al*., 1963). This selective media consists of ox bile (0.8%), NaCl (1%) and alkaline pH 8.6 to inhibit the growth of another Gram-positive pathogen. Overnight growth (18 to 24 hrs) of *V. cholerae* produces large yellow colonies with opaque centres (Figure 1A) while *V. parahaemolyticus* produce green to blue-green colonies (Figure 1B). The differences colonial morphologies of *V. cholerae* and *V. parahaemolyticus* on TCBS agar are listed in Table 2.

MacConkey agar that is commonly used to isolate members of the *Enterobacteriaceae*, is also used to grow some strains but not all strains of *V. cholerae* O1. Overnight colonies of *V. cholerae* on MacConkey agar tend to be small to moderately sized (1-3 mm) and appeared as pink colour. Suspicious colonies on

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MacConkey agar should be subjected to additional tests (antisera and/or biochemical tests) for detection of *V. cholerae*. Taurocholate-tellurite-gelatin agar (TTGA or Monsur's agar) is another choice that specifically designed for the isolation of *V. cholerae*. Overnight growth of *V. cholerae* on TTGA agar produces grey colonies with flattened opaque zone around the colony. However, this medium suffers a drawback of obtaining smaller colonies (1-2 mm) on TTGA and this medium is not commercially available.

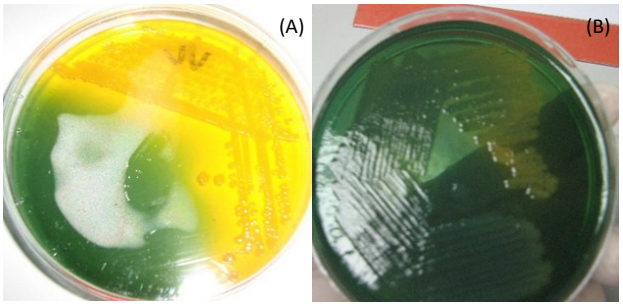

Figure 1. Colonial morphology of (A) *V. cholerae* and (B) *V. parahaemolyticus* on TCBS agar.

Table 2. Colony morphologies of *V. cholerae* and *V. parahaemolyticus* grown on TCBS agar

	V. cholerae	*V. parahaemolyticus*
Colony colour	Yellow colonies with opaque centres	Blue to green centred colonies
Morphological characteristics	Large, smooth, glistening, and slightly flattened	Convex, slightly irregular in shape, and have an undulant border
Colony size	2-3 mm	2-4 mm

CHROMagar™*Vibrio* has been used for isolation and detection of *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. cholerae*. CHROMagar™*Vibrio* is more accurate and specific than TCBS for detection of *V. parahaemolyticus* from shellfish samples (Angela *et al*., 2010). This media consists of colorimetric substrates for β-galactosidase, which was developed to identify ortho-nitrophenyl-β-galactoside-positive *V. parahaemolyticus* from other *Vibrio* pathogens. Hara-Kudo *et al.* (2001) have developed an improved and effective method for detection of *V. parahaemolyticus* in seafood using two-step enrichment procedure and subsequently plated onto a CHROMagar™*Vibrio*. Samples were first cultured in salt Trypticase soy broth (nonselective medium), followed by a portion of the culture which was subcultured with salt polymyxin broth (selective medium for *V. parahaemolyticus*). This two-step enrichments procedure was more effective in isolating *V. parahaemolyticus* than one-step enrichment in salt polymyxin broth. The enrichment cultures were finally plated onto a CHROMagar™*Vibrio* containing substrates for beta-galactosidase. This new enrichment and isolation scheme has been proved to be more sensitive and accurate for detecting *V. parahaemolyticus* in seafood samples than previously used methods.

The Wagatsuma agar was developed to differentiate of *tdh* and non-*tdh* producing strains of *V. parahaemolyticus* (Nishibuchi and Kaper 1995). The Wagatsuma agar is composed of sodium chloride, dipotassium phosphate, mannitol, crystal violet, and human or rabbit blood. *V. parahaemolyticus* strains containing *tdh* gene will produce a hemolytic halo on this agar. However, this agar cannot detect *trh* producing strains from the non-pathogenic strains, which does not cause hemolysis on the agar.

5.2 Biochemical screening tests

Oxidase test (detect the presence of cytochrome *c*) is widely used to differentiate *Vibrio* from the *Enterobacteriaceae* species. However, results might be incorrect when the colonies are obtained directly from TCBS agar or other selective medium. Thus, an additional step is required to subculture the colonies on TCBS agar by heavy inoculation onto nonselective medium prior testing for oxidase test. Kligler's iron agar (KIA) or triple sugar iron (TSI) agar was useful to rule out non-*Vibrio* species, especially *Pseudomonas* and certain *Enterobacteriaceae* species. Moreover, the string test, using fresh growth (18-24 hrs growth) and an isolated colony from non-selective agar, is useful to differentiate *V. cholerae* (string test positive) from other *Vibrio* spp. and non-*Vibrio* spp., such as *Aeromonas* spp. (Table 3). It is important to note that there is no need to use two biochemical tests to rule out the same microorganism.

Table 3. Biochemical characteristics of selected Vibrionaceae member.

Test	*V. cholerae*	*V. mimicus*	Halophilic *Vibrio*
Oxidase	+	+	+
KIA	K/A	K/A	V
TSI	A/A	K/A	V
Gas from glucose	-	-	-[a]
Sucrose	+	-	V
String	+	+	+
Lysine	+	+	V
Arginine	-	-	V

[a]*V. furnissii* and *V. damsel* are variable in gas production from glucose.
A: acid production, K: alkaline reaction, V: variable reaction

5.3 Rapid detection method

5.3.1 Immunofluorescence

Rapid laboratory identification of *V. cholerae* and *V.*

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parahaemolyticus is advantageous to monitor the spread of the disease and rapidly providing control measures. Immunofluorescence (IF) is one of the rapid detection tests that used antisera conjugated to fluorescein isothiocyanate to visualise *V. cholerae* O1 and O139 cells in a various samples types (Gustafsson and Holme 1985; Qadri *et al*., 1995). Goel *et al*. (2005) proposed that direct fluorescent polyclonal antibody staining method can be used in environmental surveillance of *V. cholerae* O1. Although immunofluorescence assay showed high specificity and no cross-reactions, but the false positive result was observed when high concentrations of Inaba specific monoclonal antibodies were used (Gustafsson and Holme 1985). Additionally, this method is not widely used as a diagnostic tool due to the requirements for high quality immunologic reagents, expensive fluorescence microscope, and trained technicians.

5.3.2 Molecular identification

Bacterial culture has long been recognised as the "gold standard" for the detection of laboratory diagnosis of cholera. However, this traditional method is inadequate for rapid diagnosis due to lengthy culturing on selective growth media, highlighting the overarching neglect of field diagnostic requirements. Moreover, the conventional phenotyping and biochemical detection are complicated when they are isolated from seafood and aquatic environment.

Molecular techniques such as nucleic acid-based methods have been developed to overcome these limitations and to improve the detection of *Vibrio* pathogens. *Vibrio* species are well known to contain highly variable genetic composition. Thus, those specific genes present in *Vibrio* strain can be candidates for the development of DNA-based marker for molecular detection. Polymerase chain reaction (PCR), a molecular detection method, was widely used for the detection of *Vibrio* pathogens in various samples including seafood and water samples. This detection method can be applied because of the presence of several conserved genes, including, RTX (repeat in toxin) toxin gene (Chow *et al*., 2001), *toxR* gene (Kim *et al*., 1999) and *gyrB* gene (Venkateswaran *et al*. 1998). The RTX toxins are important virulence factors that found in Gram-negative bacteria (Coote 1992). Two of the RTX toxin genes: *rtxA* and *rtxC* were potentially used as gene markers to specifically detect *V. cholerae* since they are not identified in *V. parahaemolyticus*, diarrheagenic *Escherichia coli, Aeromonas* species and *Plesiomonas* species (Chow *et al*., 2001). Multiplex PCR assays are very popular and being used to differentiate *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* from each other as well as from other *Vibrio* species (Di Pinto *et al*., 2005; Wei *et al*., 2014). Recently, a cost effectiveness multiplex PCR assay was developed using virulence- and toxigenic-associated (VTA) genes (*ctxA*, *tcpA* and *ompW*) to differentiate *V. cholerae* from other *Vibrio* species and other bacteria from the *Enterobacteriaceae* family (Mehrabadi *et al*., 2012). This assay can detect 10-100 CFU of *V. cholerae* or 8.5-85 pg genomic DNA (Mehrabadi *et al*., 2012). Saha *et al*. (2006) developed a chromosomal replication origin (ori) sequence-based PCR-restriction fragment length polymorphism (RFLP) assay for rapid identification and differentiation of *V. cholerae* and their closely related species *V. mimicus*. This assay is also useful for the differentiation between classical and El Tor biotypes of *V. cholerae* O1 (Saha *et al*., 2006).

For detection of *V. parahaemolyticus*, *toxR* and *gyrB* genes were widely used for detection using PCR because these genes are well conserved among *V. parahaemolyticus* isolates (Venkateswaran *et al*., 1998; Kim *et al*., 1999). Alternatively, the thermostable direct hemolysin (*tdh*), TDH related hemolysin (*trh*) and thermo labile hemolysin (*tlh*) in *V. parahaemolyticus* are another genes that were used to develop a multiplex PCR for concurrent detection of total (*tlh* gene) and virulent (*tdh* and *trh* genes) *V. parahaemolyticus* in shellfish samples (Bej *et al*., 1999). Although the *tlh* gene is not a virulence factor of *V. parahaemolyticus* (Su and Liu 2007), it is a reliable gene marker for the detection of *V. parahaemolyticus* strains isolated from various samples, such as seafood, environmental, oyster plants, and clinical samples (Bej *et al*., 1999). In 2006, a multiplexed real-time PCR TaqMan assay was developed to detect four different genes (*tlh*, *tdh*, *trh* and *ORF8*) in total and virulent *V. parahaemolyticus*, including pandemic O3:K6 serotype (Ward and Bej 2006). More advanced loop-mediated isothermal amplification (LAMP) based technology was initially used to detect *tdh*-, *trh*, and *tlh*-positive isolates of *V. parahaemolyticus* (Yamazaki *et al*., 2008; Yamazaki *et al*., 2010). LAMP assay requires lesser bacteria for detection compared to conventional PCR assay. Another higher sensitivity LAMP assay was also successfully developed to target *rpoD* and *toxR* genes of *V. parahaemolyticus* (Nemoto *et al*., 2011).

6. Risk factor and prevention

Several clinical cases and outbreaks caused by *V. cholerae* and *V. parahaemolyticus* have been reported previously in Malaysia. These cases were strongly associated with seafood consumption in Malaysia. In Malaysia, these foodborne disease outbreaks mainly occur due to unhygienic and insanitary food handling

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method (post-harvest and food preparation handling) which accounted for more than 50% of the poisoning cases. In most cases, high contamination levels of *Vibrio* spp. were caused by high initial levels of the pathogens in seafood while subsequent inappropriate (poor hygiene and sanitation) storage conditions of the seafood by retailers may further promote their growth up to hazardous levels. In addition, inappropriate food handling practices, meals prepared in advance and food was kept at ambient temperature until served is also considered as the dominant risk factor (Sharifa *et al*., 2013).

Therefore, in order to prevent vibriosis and cholera, it is important to pay attention to temperature control during post-harvest handling of the seafood. After harvest, *Vibrio* species such as *V. parahaemolyticus* can multiplied rapidly in live seafood held at ambient temperature (25-26°C), where it has been shown that *V. parahaemolyticus* can increase by 50-fold after 10 h of post-harvest storage at 26°C (Gooch *et al*., 2002). Parveen *et al*. (2013) have demonstrated that *V. parahaemolyticus* would be slowly inactivated at 5 and 10°C with average rates of -0.002 and -0.001 log CFU/ hr, respectively (Parveen *et al*., 2013). This was supported by Gooch *et al*. (2002) who reported that average *V. parahaemolyticus* counts showed a six-fold decrease (0.8 log CFU/g) after approximately 14 days of refrigeration. Liu *et al*. (2009) investigated the effect of frozen storage of oyster after harvesting and found that the populations of *V. parahaemolyticus* in the oysters declined by 2.45 log MPN/g after 1 month and 4.55 log MPN/g after 6 months of storage at -10°C. Although cold storage has been shown to reduce the load of *Vibrio* spp., consumption of raw seafood and/ or seafood products should still be avoided.

Care should be taken during preparation of seafood by ensuring adequate cooking before consumption will also eliminate or drastically reduce the incidence of foodborne gastroenteritis caused by *Vibrio* spp. (Sani *et al*., 2013). *V. parahaemolyticus* is heat sensitive with D_{52} value of 1.3-1.5 mins as tested in oyster (Andrew *et al*., 2003). The author also suggested that a total processing time of at least 22 mins at 52°C was recommended to reduce this bacterium to non-detectable levels. In another study, a heat treatment of 70°C for 2 mins was found to be effective against *V. cholerae* and *V. parahaemolyticus* in shrimp (Johnston and Brown 2002).

Interestingly, Mathurand and Schaffner (2013) has demonstrated that marination of raw fish with lime juice can potentially reduce *V. parahaemolyticus* levels by 5 log. This was probably due to the presence of organic acids and polyphenol compounds in lime juice that exhibit antimicrobial effects. Utilisation of fresh squeezed lemon juice (diluted to 1%) can effectively inhibit *V. cholerae* (10^8 CFU/mL) by 100% after 5 min of exposure time. This shows that natural products such as lime and lemon which is easily found and harmless to humans could act as an effective biocide against *Vibro* spp. (de Castillo *et al*., 2000). In addition to food preparation/ processing, cross-contamination during handling and post-cooking must also be taken into consideration to reduce the incidence of *Vibrio* spp. related gastroenteritis.

Furthermore, the prevalence of *Vibrio* spp. in seafood can also be controlled by probiotics treatment during farming. Vaseeharan and Ramasamy (2003) has demonstrated that treatment of black tiger shrimp (*Penaeusmonodon*) with *Bacillus subtilis* BT23 (7-9 log CFU/mL) significantly reduced the *Vibrio* spp. by 5-7 log CFU compared to shrimp without probiotics treatment. In agreement, probiotic bacteria have also been demonstrated to control Vibriosis in fish, Tiger Grouper Fry (*Epinephelus fuscoguttatus*) (Ilmiah *et al*., 2013). The addition of probiotics reduced the pathogenic *Vibrio* population in the fish by more than 50% after 24 hrs treatment and significantly increased the survival rate of the fish after 28 days of treatment. Although antibiotics have been commonly used in aquaculture to prevent Vibriosis, probiotic treatment may offer a promising alternative to the use of antibiotics in shrimp and fish aquaculture. Utilisation of probiotics not only suppresses the population of *Vibrio* pathogens but also increased the survival rate of fish and other seafood. This subsequently increases the fisheries production and provides a safer food supply for consumers. However, the exact mechanism of action for the antimicrobial effect of probiotics treatment remains unclear. Thus, further studies are deemed necessary to investigate the exact mode of action and limitation of such approach for microbial control in aquaculture. The summary of prevention and control measures that could be taken to control *Vibrio* spp. in food supply chain is illustrated in Figure 2.

The health effect caused by *V. parahaemolyticus* is not severe, usually not life threatening, short duration and symptoms are self-limiting. Similarly, the disease caused by cholera could be quite mild with symptoms including sudden onset of profuse watery diarrhoea, muscle cramp, and vomiting. Infected persons may be treated with antibiotics to enhance recovery, along with electrolytes to replace the body fluids loss. Unexposed people can also be vaccinated to protect them from cholera.

7. Socio-economic impact

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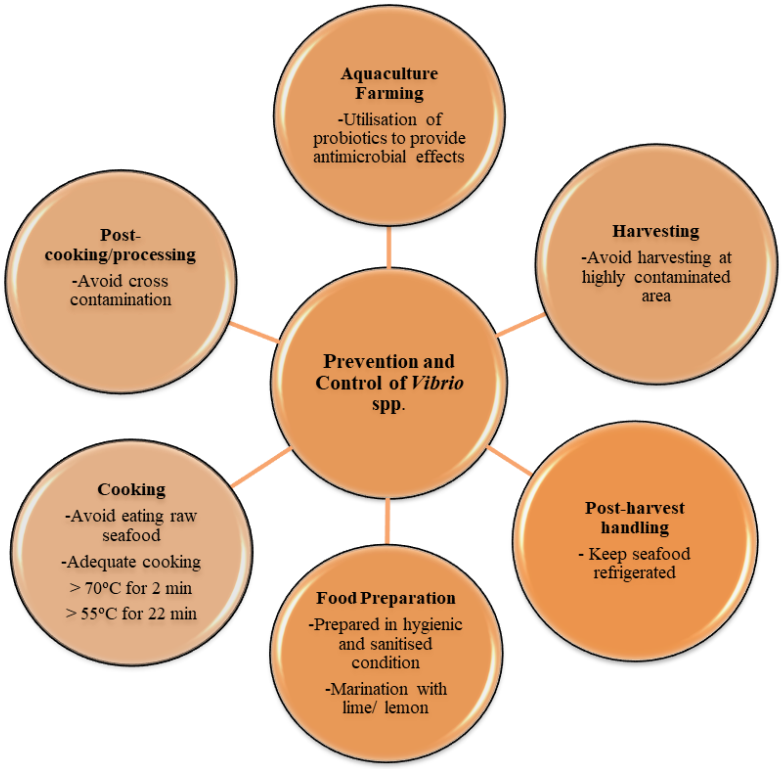

Figure 2. Prevention and control of *Vibrio* spp. in food from farming to post processing handling.

In general, outbreaks caused by *V. cholerae* and *V. parahaemolyticus* may affect human health and lead to human suffering. In addition to human suffering, cholera outbreaks also cause panic, disruption of the social and economic structure, and leading to obstruction of development in the affected communities. In certain cases, the outbreak may also cause unwarranted panic-induced reactions by other countries including restricting travel from countries where an outbreak is occurring or restriction on import of certain foods from the affected countries. The disease caused by *Vibrio* in seafood (Vibriosis) also caused mass mortality in the cultivation of fisheries, resulting in a huge economic loss. For instance, in 1992, the marine finfish cage culture industry experienced a tremendous economic loss when the main cultured species, mainly groupers and sea bass, were suspected to be infected with Vibriosis. During this period, a cage fish farmer in Penang was reported to loss approximately RM 3,000 a day due to Vibriosis (FAO 1995). All these will lead to detrimental economy and trade consequences.

8. Conclusion

Vibrio species are important enteric pathogenic bacteria that causing foodborne infection. *Vibrio cholerae* (O1 and non-O1 serogroup) and *V. parahaemolyticus* have been implicated in foodborne illness. The presence of *V. cholerae* and *V. parahaemolyticus* in marine environment raises concerns about food safety as seafood remains as the main vehicle of transmission for these pathogens. Proper sanitation, heat treatment, and refrigeration can be used to reduce their incidence. In Malaysia, although *Vibrio* did not produce a high rate of mortality, effective control measures, molecular surveillance and epidemiology are still required to ensure the safety of food as well as to reduce the risk of infection caused by these pathogens.

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