



Consumption of raw oysters: a risk factor for *Vibrio parahaemolyticus* infection

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Article history

Received: 14 June 2014

Received in revised form:

10 August 2014

Accepted: 11 August 2014

Keywords

Vibrio parahaemolyticus

MPN-PCR

Microbial Risk Assessment

Antibiogram

Abstract

Vibrio parahaemolyticus is recognized as a frequent causal agent of human gastroenteritis due to the consumption of raw, undercooked or mishandled seafood in many Asian countries. The number of *V. parahaemolyticus* cases reported is on the rise, and this becomes a concern to the Asian countries as seafood is favoured by Asians. This study aimed to detect and quantify *V. parahaemolyticus* in raw oysters and to determine the risk associated with the consumption of raw oysters. A total of 30 oyster samples were collected and analysed in this study. MPN-PCR and MPN-Plating methods were employed and carried out concurrently to determine the prevalence of *V. parahaemolyticus* in raw oysters. The results showed that the prevalence of total *V. parahaemolyticus* in oysters was 50.00% (15/30) where the MPN/g range was $< 3 - > 11000$ MPN/g for MPN-PCR method, and 40.00% (12/30) where the MPN/g range was $< 3 - 4300$ MPN/g for MPN-Plating method. MPN-PCR method was able to estimate the level of virulence (tdh⁺ and trh⁺) *V. parahaemolyticus* in the raw oysters where 10.00% (3/30) of samples were identified to be in a range of 3 – 30 MPN/g. A microbial risk assessment was conducted based on the enumeration data obtained from MPN-PCR method using @risk. The probability of illness annually was 1.76×10^{-6} with a prediction of 31 cases to occur with respect to the exposed Malaysian population, while the rate per 100,000 people was estimated to be at 0.104. In addition, the antibiogram of *V. parahaemolyticus* was determined using Kirby Bauer Disk Diffusion Test and the results indicated that the isolates were highly resistant towards Bacitracin (100.00%), Vancomycin (100.00%) and were least resistant to Chloramphenicol (8.70%). The MAR index of the isolates ranged from 0.17 to 0.50. In accordance with the results from this study, the consumption of raw oysters is a risk factor for *V. parahaemolyticus* infection and proactive actions should be taken to reduce the risk of the pathogen in order to improve public health.

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Introduction

The safety level of seafood consumption has become a concern to the public as seafood that provides easily digested protein, is favoured by many individuals. Incidences of *Vibrio parahaemolyticus* cases were reported to rise by 43% in 2012 compared to the period from 2006 to 2008 by Central of Disease Control (CDC) (Food Safety News, 2013). *V. parahaemolyticus* has made its mark in the United States as the leading causative agent of human gastroenteritis from the consumption of raw or mishandled seafood. Numerous cases of outbreaks were linked to the consumption of oysters as they are bivalve mollusks and, commonly eaten raw. Due to that reason, consumers are highly exposed to the risk of foodborne pathogens, especially *V. parahaemolyticus* that is the normal microflora found in marine and estuarine environments in almost all

temperate regions (Chang *et al.*, 2011). In summer 2006, about 177 cases reported in Washington and British Columbia were linked to the consumption of harvested contaminated oysters (CDC, 2009).

On the other hand, Liu *et al.* (2009) reported that *V. parahaemolyticus* infection cases increased significantly in Asia. *V. parahaemolyticus* has partially become one of the sources of half of the foodborne outbreaks in Asian countries (Solomakos *et al.*, 2012). Between 1998 and 2007, *V. parahaemolyticus* was one of the etiological agents of the large-scale outbreaks in Japan where a total of 5 cases were reported (Infectious Agents Surveillance Report [IASR], 2008). *V. parahaemolyticus* accounted for 78.4% (1999-2008) and 17.1% (2006-2010) of all bacterial pathogens causing foodborne illness in Taiwan and Korea, respectively (Chang *et al.*, 2011; Kim *et al.*, 2012). In Hat Yai, Thailand, 319 sporadic cases of *V. parahaemolyticus* were reported due to the

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consumption of bloody clams (FAO/WHO, 2011). Recently, 49 cases of *V. parahaemolyticus* with acute diarrhoea were reported in Tbaung Boeung's Village, Cambodia due to the consumption of raw octopus (Vandy *et al.*, 2012).

V. parahaemolyticus is a Gram-negative, halophilic and non-spore forming bacterium. According to Iida *et al.* (1998), environmental *V. parahaemolyticus* is mostly non-pathogenic. However, a sub-population of *V. parahaemolyticus* was identified as pathogenic harbouring the *tdh* gene or the *trh* gene encoded as TDH and TRH respectively, which had been the causative agent of foodborne illness related to the consumption of raw or undercooked seafood (Honda and Iida, 1993; Nishibuchi and Kaper, 1995; FDA, 2012). TDH is an enzyme toxin that has the ability to lyse red blood cells while TRH is a putative virulence factor that plays a similar role in the pathogenesis of *V. parahaemolyticus* as reported by Shirai *et al.* (1990). The amino acid sequence of TRH is approximately 67% identical to TDH with similar biological activities such as haemolytic activity, cytotoxicity, cardiotoxicity and enterotoxicity (Honda and Iida, 1993). Emerging strain of *V. parahaemolyticus* serotype O3:K6 have been reported to cause gastroenteritis in many countries since 1996. This new, highly virulent strain known to possess *tdh* gene, has now rapidly disseminate globally, resulting in an increase of *V. parahaemolyticus* infection (Ansaruzzaman *et al.*, 2005; Parveen and Tamplin, 2013). Infection by pathogenic *V. parahaemolyticus* can cause development of acute gastroenteritis characterized by diarrhoea, headache, vomiting, nausea, abdominal cramps and low-grade fever. It is often self-limiting, but the infection may cause septicaemia in severe cases that are life-threatening in immunocompromised individuals.

The traditional method of isolation and enumeration of *V. parahaemolyticus* involving the most probable method (MPN) coupled with traditional confirmation techniques had several drawbacks due to the large amount of workload, material and time needed (Nishibuchi, 2006). As a result, the development of molecular methods involving polymerase chain reaction (PCR) for detection of pathogens were introduced, and proven to provide quantitative and qualitative determination of the organism (Su and Liu, 2007). MPN coupled PCR was proven to be significantly time saving, labour saving and the results can be obtained within two days as stated by Martin *et al.* (2004). The quantitative determination had enabled some researchers to further conduct microbial risk assessment as a tool to manage food safety risks and reduce the impact of

disease-causing pathogens.

Thus, the study aimed to detect and quantify *V. parahaemolyticus* in raw oysters using the MPN-PCR method and the MPN-Plating method for comparison purpose. A microbial risk assessment was conducted by using the quantitative data from the MPN-PCR method to determine the risk associated with the consumption of raw oysters. Based on the epidemiology of consumption of raw oysters, it is necessary to provide a collection of data in Asia to monitor the risk posed by *V. parahaemolyticus*. The antibiogram of *V. parahaemolyticus* isolated from raw oysters was determined as well.

Materials and Methods

Sampling

A total of 30 raw oyster samples were randomly purchased from local supermarkets in Kuala Lumpur and Selangor. They were transported in portable coolers at ambient temperature to the laboratory and analysed upon arrival.

Most probable number (MPN) procedure

The MPN procedure was carried out by following the protocol described by Bacteriological Analytical Manual with minor modifications (Kaysner *et al.*, 2004). Approximately 10 g portion of the sample was weighed into a sterile stomacher bag and 90 ml of alkaline peptone water broth (APW) (1% peptone [Oxoid]; 1% NaCl [Merck, Germany]) was added. The mixture was stomached for 60 seconds using a stomacher (Interscience, France). The stomached mixture was then diluted 10-fold for four successive times, and pre-enriched at 37°C for 24 hours prior to Most Probable Number (MPN) analysis. For MPN analysis, 1 ml of each mixture was transferred into three tubes set containing 9 ml of APW and incubated at 37°C for 24 hours. Turbid MPN tubes were then streaked onto Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar (Eiken Chemical) for confirmation. The agar plates were incubated at 37°C for 18 to 24 hours. *V. parahaemolyticus* positive tubes were determined based on the isolation of green centered colonies on TCBS agar plates. All MPN tubes were subjected to DNA extraction to enable PCR detection.

Boiled-cell DNA extraction method

One ml of the culture mixture was transferred from the MPN tubes into the micro centrifuge tubes and centrifuged at 10,000 rpm for 3 minutes to pellet the microorganisms. The supernatant was discarded and 200 µl of sterile distilled water was added. The resulting mixture was vortexed to ensure that the

Table 1. Primers used in multiplex PCR detection of *V. parahaemolyticus*

Target	Primer	Sequence 5' → 3'	Amplicon, bp
<i>trh</i>	<i>trh</i> -F	TGGGCTTCGATATTTTCAGTATCT	484
	<i>trh</i> -R	CATAACAACATATGCCATTTCGG	
<i>toxR</i>	<i>toxR</i> -F	GTCTTCTGACGCAATCGTTG	368
	<i>toxR</i> -R	ATACGAGTGGTTGCTGTCATG	
<i>tdh</i>	<i>tdh</i> -D3	CCACTACCACTCTCATATGC	251
	<i>tdh</i> -D5	GGTACTAAATGGCTGACATC	

pellet dissolved. Next, the micro centrifuge tubes were subjected to boiling temperature at 105°C for 10 minutes using a dry cell bath (Labnet International, Inc.) and stored at -20°C until further use for PCR detection as a template. Presumptive single colonies of *V. parahaemolyticus* on the TCBS agar were picked randomly and suspended in Tryptic Soy Broth (Merck, Germany). The culture mixture was then subjected to the boiled-cell DNA extraction method as briefly aforementioned and used as a template for the confirmation of *V. parahaemolyticus* using PCR.

Polymerase chain reaction (PCR)

PCR amplification was performed in a 25.4 µl of reaction mixture containing 12 µl of sterile distilled water; 7 µl 10X PCR Buffer (Promega, USA); 2 µl magnesium chloride (MgCl₂) (Promega, USA); 0.5 µl of deoxyribonuclease triphosphate (dNTP) mix (Vivantis, Malaysia); 0.5 µl of 10 mM of primer mix containing each primer; 0.4 µl of *Taq* Polymerase (Promega, USA) and 3 µl of DNA template solution. The primers used in the multiplex PCR for the detection of *V. parahaemolyticus* are as shown in Table 1. Multiplex PCR was employed to amplify the reaction mixture using a 96 Well Thermal Cycler (Applied Biosystems, Veriti). The reaction mixture was heated at 95°C for 3 minutes for initial DNA denaturation. Following next, 35 repetitive cycles were carried out. Each cycle comprises of three different temperatures, which were 95°C for 30 seconds, 60°C for 45 seconds and 68°C for 60 seconds for denaturation, amplification and elongation respectively. Then, holding took place at 72°C for 3 minutes before termination.

Agarose gel electrophoresis

The PCR products were visualized using agarose gel electrophoresis. 1.25% of agarose gel was prepared and stained with 1 µl of ethidium bromide. Subsequently, 5 µl of the PCR products was pipetted into the well and the gel electrophoresis was run at 100 V for 30 minutes in the Tris-borate-EDTA running buffer. The agarose gel was then viewed under ultra-violet light (GeneSnap, SynGene). Identification of strains was done by targeting the presence of regulatory *toxR* gene as described by Kim *et al.* (1999) while virulent strains were identified by targeting the presence of *tdh* gene and *trh* gene as

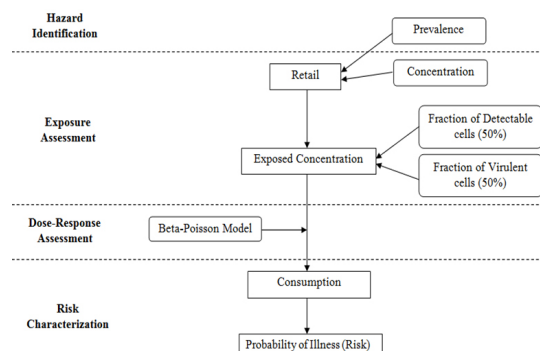


Figure 1. Model structure of risk assessment of *V. parahaemolyticus* in raw oysters in Malaysia from retail stage to consumption

described by Tada *et al.* (1992) and Bej *et al.* (1999), respectively.

Microbial risk assessment

The @risk software package (Palisade Corporation, USA) in combination with Microsoft Excel was used to run the simulations. The Monte Carlo method was adopted in performing the calculations and a total of 10,000 iterations were undertaken. The prevalence of total and virulent (*tdh*⁺ and *trh*⁺) *V. parahaemolyticus* strains obtained from MPN-PCR method were used as input data generated in this risk assessment. Thus, it was used to calculate the risk estimate along with other information required.

The model structure was constructed as shown in Figure 1. The model was constructed at retail to consumption on the assumption that the detectable and virulent *V. parahaemolyticus* remained the same. In this model, fractions of detectable and virulent cells were calculated and the assumption was made that there was a 50:50 chance of consumption of detectable and virulent cells that can cause acute gastroenteritis. The likelihood of illness following infection cases from consumption of oysters per annum, based on the exposed population in Malaysia was calculated.

Exposure assessment

(i) Contamination level of *V. parahaemolyticus* at retail

Contamination level of *V. parahaemolyticus* at retail was assessed separately for detectable and virulent *V. parahaemolyticus* using the same parameters and models. The following explains the methodology of the model used for the assessment. The original contamination rate (P_p) of *V. parahaemolyticus* was estimated using a Beta ($s+1$, $n-s+1$) function in @risk. The contamination level of positive *V. parahaemolyticus* (LP) was modelled using a lognormal distribution with mean

Table 2. Description of parameters and model for exposure assessment of *V. parahaemolyticus* in raw oysters

Parameters	Description of Parameters	Input Model	Input Values	
			Detectable	Virulent
P_p	Original <i>V. parahaemolyticus</i> contamination rate	Beta (s+1, n-s+1) ^a	Beta (16, 16)	Beta (4, 28)
1- P_p	Non-detectable <i>V. parahaemolyticus</i> contamination rate			
L_p	Contamination level of positive <i>V. parahaemolyticus</i>	Normal (μ , σ) ^b	Normal (1.902, 1.347)	Normal (1.105, 0.547)
L_n	Non-detectable level of <i>V. parahaemolyticus</i>	Equation (1)	-1.159 log MPN/g	-1.977 log MPN/g
N_0	Contamination level of <i>V. parahaemolyticus</i> at retail	Uniform (min, max) ^c	Uniform (-2.795, 0.477)	Uniform (-4.431, 0.477)
C_e	Customer Exposure	Discrete (L_p : L_n : P_p :1- P_p) Discrete (N_{0D} : N_{0V} :0.5:0.5) ^d		

^a s = number of positive samples, n = total number of samples

^b μ = mean value in log10, σ = standard deviation value in log10

^c The min and max are the respective values calculated from equation 1 in log10

^d N_{0D} = Contamination level of detectable *V. parahaemolyticus* at retail

N_{0V} = Contamination level of virulent *V. parahaemolyticus* at retail

and standard deviation values of the MPN/g data. This study adopted 'fail-safe' conditions as explained by Jarvis (2000). The condition inferred as non-detectable level of *V. parahaemolyticus* in negative samples may indicate the true absence of the bacteria or the level of detection is below the detection limit. Due to this, equation (1) developed by Jarvis (2000) was used to calculate the non-detectable level of *V. parahaemolyticus* (L_n) using the Uniform distribution.

$$M = -\left(\frac{2.303}{V}\right) \log \frac{Z}{N} \quad (1)$$

Where M is the true density in the batch; V is the quantity of the material examined; Z is the number of negative samples detected while N is the total number of samples examined. Using the equation, the calculated M value will be used as an average non-detectable level of *V. parahaemolyticus* in negative samples and by referring to the data in Table 4, the minimum and maximum values were calculated as the input values of the uncertainty. The contamination level of *V. parahaemolyticus* (N_0) at retail will be calculated by using the discrete distribution. The description of parameters and models for exposure assessment are summarized in Table 2.

(ii) Dose exposed to consumer

The model assumes that the level of *V. parahaemolyticus* remained constant at retail when purchased to consumption as oysters are consumed raw with no cooking methods carried out prior to consumption. The model also adopted the assumption that consumers are exposed to a 50:50 ratio of detectable and virulent *V. parahaemolyticus* and have the possibility to suffer from adverse health effects. Thus, the dose exposed to consumers was calculated on a 50:50 ratio of detectable and virulent *V. parahaemolyticus* using discrete distribution (Table 2).

Hazard characterization

The probability of illness (P_{ill}) per meal was estimated using the wide usage Beta-Poisson model proposed in the United States Food and Drug

Administration (US FDA) as shown in the equation below.

$$P_{ill} = 1 - (1 + D/\beta)^{-\alpha}$$

Where D is the dose and α and β are parameters corresponding to the beta distribution parameters for a specific range. Values of α and β were adopted from the feeding test data of 20 subjects conducted by US FDA which fits the Beta-Poisson model for *V. parahaemolyticus*. Infection by *V. parahaemolyticus* is characterized by an acute gastroenteritis with symptoms such as abdominal cramps, explosive watery diarrhoea, nausea, vomiting and sometimes fever. Symptoms have often ranged from mild to severe, but self-limiting (Lake *et al.*, 2003).

Risk characterization

The qualitative or quantitative information is gathered and interpreted in risk characterization. When solved, it provides the risk estimate interpreted as the probability of an adverse effect. The probability of illness yearly was calculated based on the equation below as described by Robertson *et al.* (2005).

$$P_{ill}/\text{year} = 1 - (1 - P_{ill}) \text{ no. of serv per year}$$

In this model, it was assumed that the Malaysian population consumed 365 servings of oysters per year. The number of expected cases to occur was calculated by multiplying the exposed population with the probability of illness yearly. As reported by MOH Malaysia (2003), 58.81% of the Malaysian population consumed seafood, and was regarded as the exposed population in this model.

Antibiotic susceptibility test

Each isolate of *V. parahaemolyticus* was tested with 12 different antibiotics [Imipenem (10 μ g), Norflaxin (10 μ g), Erythromycin (15 μ g), Bacitracin (10 μ g), Kanamycin (30 μ g), Gentamicin (10 μ g), Chloramphenicol (30 μ g), Clarithromycin (15 μ g), Amikacin (30 μ g), Vancomycin (30 μ g), Nalidixic Acid (30 μ g), Streptomycin (10 μ g)] based on the

standard method by Bauer *et al.* (1966) on Mueller-Hinton agar (Merck, Germany). The cultures were grown in Luria-Bertani, LB Broth at 37°C for 18 to 24 hours and were swabbed onto dry Mueller-Hinton agar plates using sterile cotton swabs. The agar plates were allowed to dry for 5 to 10 minutes. After that, antibiotic discs were dispensed onto each plate with sufficient space between the discs. The plates were then inverted and incubated at 37°C for 18 to 24 hours. The diameter of the inhibition zones on the discs was measured and recorded in millimeters (mm). The zone of inhibition was interpreted into three different categories which are resistant (R), intermediate (I) and susceptible (S) by referring to the CLSI M100 S23 2013 Disc Diffusion Supplemental Table.

Multiple antibiotic resistant (MAR) index

Based on the occurrence of the multiple resistances of isolates, the results were quantified using the MAR index. The MAR index of the isolate is defined as a/b where 'a' represents the number of antibiotics to which the particular isolate is resistant and 'b' represents the number of antibiotics to which the isolate is exposed (Krumperman, 1983).

Results and Discussion

Prevalence

Prevalence of *V. parahaemolyticus* in oysters is well documented in most of the countries such as Japan, Taiwan, Korea and the United States that have experienced vibriosis outbreaks from eating raw oysters. As oysters are bivalve mollusks which are filter feeders, they feed by filtering the suspended food particles from the coastal shores. This includes trapping various microorganisms present in the marine sea. As a result, they commonly serve as carriers for pathogens, especially members of the family Vibrionaceae. Apart from *V. parahaemolyticus*, oysters are also considered as the source of infection for all other vibrios as well.

The results of the experiment indicated that the presence of *V. parahaemolyticus* in the oyster samples was inevitable. The employment of MPN-PCR and MPN-Plating methods were able to detect and quantify the organism. The MPN/g of *V. parahaemolyticus* in a total of 30 samples analysed ranged from <3 to >11000 MPN/g by using MPN-PCR method and was relatively lower for MPN-Plating method that ranged between <3 and 4300 MPN/g. The MPN-PCR method was able to detect 15/30 (50.00%) positive samples compared to the MPN-Plating method which only detected 12/30 (40.00%) positive samples. From the results, the broad range of MPN/g suggests a high

possible risk of infection of *V. parahaemolyticus*. The high MPN/g levels of *V. parahaemolyticus* recorded in four raw oyster samples indicated that the growth *V. parahaemolyticus* had reached to the infectious level. According to FAO/WHO, the established limit of *V. parahaemolyticus* imposed for oysters are between 10^2 to 10^3 MPN/g and it is rather clear that the raw oysters were unfit for consumption.

High contamination levels of *V. parahaemolyticus* were caused by high initial levels of *V. parahaemolyticus* in oysters. The poor hygiene and sanitation of the storage conditions of the live oysters by the retailers had contributed to the growth of *V. parahaemolyticus* up to hazardous levels from its initial contamination level. At the same time, cross-contamination among the live oysters will occur. The water temperature also plays a role in the distribution of *V. parahaemolyticus* in the environment and oysters as stated by DePaola *et al.* (1990). Besides that, it also affects the pathogenicity of *V. parahaemolyticus* strains. Goertz *et al.* (2013) stated that virulent strains may be associated with cold tolerance. The minimum growth temperature of *V. parahaemolyticus* is at 13 to 15°C (Kim *et al.*, 2012; Goertz *et al.*, 2013). Thus, when the temperature rises above 15°C, proliferation of *V. parahaemolyticus* occurs rapidly up till a hazardous level with a higher population of pathogenic strains.

Due to its water temperature sensitivity at 15°C, *V. parahaemolyticus* is considered as an indicator of climate change. The physical and biological ocean characteristics are currently being altered due to climate change, which consequently alters the distribution and pathogenicity of marine organisms (McLaughlin *et al.*, 2005; Baker-Austin *et al.*, 2012; Parkinson and Butler, 2012). Increased sea surface temperatures and decreasing salinity of coastal waters due to increased freshwater input ultimately optimize environmental conditions for *V. parahaemolyticus* to proliferate. Additionally, impacts on other environmental parameters such as ocean acidification lead to more virulent strains of existing pathogens and changes in their distribution, or the emergence of new pathogens. According to the CDC (2010), increased acidity of water affects the formation of the molluscan shellfish carbonate shells and immune response. Eventually, this makes them more vulnerable to microbial infections. Consequently, the compounding effects of climate change will cause a range of pathogenic infections, which makes consumption of molluscan shellfish, particularly oysters a public health concern.

It is also noted that the cohabitation of *V. parahaemolyticus* with other microorganisms may

Table 3. Quantification data from MPN-PCR method of positive detectable and virulent *V. parahaemolyticus* in raw oysters

Positive Samples of <i>V. parahaemolyticus</i>	Detectable (MPN/g)		Virulent (MPN/g)	
	<i>toxR</i>		<i>tdh+</i>	<i>trh+</i>
A	150		-	-
B	3		-	-
F	110		-	-
G	3		3	-
J	3.6		-	-
K	23		-	-
M	23		-	23
O	23		-	-
P	2900		30	-
U	23		-	-
V	6.2		-	-
W	9.4		-	-
AB	>11000		-	-
AC	>11000		-	-
AD	>11000		-	-

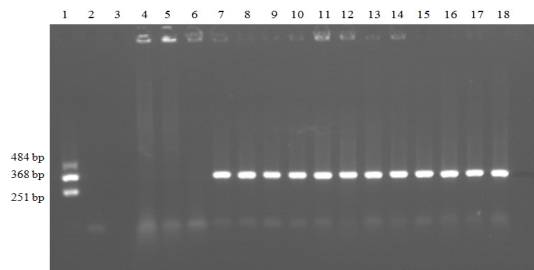


Figure 2. A representative amplification of the *toxR* gene of *V. parahaemolyticus*. Lane 1 shows the positive control with molecular size markers (*trh* gene at 484 bp; *toxR* gene at 368 bp; *tdh* gene at 251 bp). Lane 2 shows the negative control. Lane 3 shows blank. Lanes 4 to 6 are MPN tubes that tested negative at 10^{-1} dilution. Lane 7 to 18 are MPN tubes that tested positive following the dilution 10^{-2} (Lane 7-9), 10^{-3} (Lane 10-12), 10^{-4} (Lane 13-15), 10^{-5} (Lane 16-18).

increase the survival and growth of the former in oysters. A study by Lopez-Joven *et al.* (2013) on the uptake and retention of non-pathogenic *V. parahaemolyticus* by other microorganisms indicated the possibility of high and stable contamination of *V. parahaemolyticus* through cohabitation with other microorganisms in bivalve mollusk. Furthermore, the cohabitation of microorganisms will tend to induce the formation of biofilm, a protective matrix, which are exopolysaccharides (EPS) secreted by bacteria that can form a bound capsule layer when associated with the cell wall or released by the cell to create a matrix structure (Leigh and Coplin, 1992). Biofilm is adherent to an inert or a living surface and, is produced by most bacteria including *V. parahaemolyticus*. This puts the consumption of raw oysters at higher risk.

By using the MPN-PCR method, the positive identification of *V. parahaemolyticus* was determined by the presence of amplified *toxR* gene as shown in Figure 2. Kim *et al.* (1999) stated that all strains of *V. parahaemolyticus* harbour the *toxR* gene that is involved in the regulation of many genes. The specificity of PCR to amplify the *toxR* gene in *V. parahaemolyticus* had enabled high sensitivity

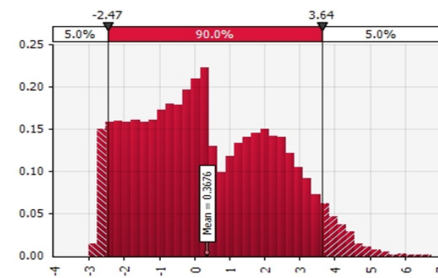


Figure 3. Simulated distribution of contamination of detectable *V. parahaemolyticus* in raw oysters at retail in log MPN/g

detection of this organism either pathogenic or non-pathogenic in a pool of microorganisms (Sechi *et al.*, 2000). This study was in agreement with Miwa *et al.* (2003) and Nordstrom *et al.* (2007) that the MPN-PCR method was more reliable in estimating the total levels of *V. parahaemolyticus*. When compared to the MPN-Plating method, the MPN-PCR method was indeed more reliable, sensitive and accurate. The reasons that contributed to the lack of estimation in the MPN-Plating method include decreased sensitivity of the selective and enrichment media for natural populations, the presence of other background bacteria including other vibrios, and the ability of *V. parahaemolyticus* to enter viable, but non-culturable state under extreme environmental conditions (Alam *et al.*, 2002; Oliver, 2005; Luan *et al.*, 2008).

PCR method is often been selected as the main choice for the detection and monitoring programs of foodborne pathogens (Vickery *et al.*, 2003; Nishibuchi, 2006). Nakaguchi (2013) added that this method was useful for surveys and epidemiological analysis of pandemic strains. It is recommended for faster detection in controlling foodborne pathogens. More importantly, the usage of MPN-PCR method allowed the levels of virulent *V. parahaemolyticus* to be estimated from total *V. parahaemolyticus* levels (Miwa *et al.*, 2003; Nordstrom *et al.*, 2007). It permitted simultaneous examination of thousands of *V. parahaemolyticus* cells of *tdh* and *trh* genes from each MPN tube. From Table 3, it can be observed that 3/30 (10.00%) samples harbour virulent strains where 2/30 (6.67%) consists of the *tdh* gene and 1/30 (3.33%) consists of the *trh* gene. Virulent strains of *V. parahaemolyticus* ranged from 3 to 30 MPN/g in the 30 raw oysters sample analysed. The total number of raw oysters contaminated with virulent *V. parahaemolyticus* were relatively low in this study. Studies conducted in the United States suggested that potentially pathogenic strains are isolated less frequently from the environment and food. Pathogenic strains appear to constitute approximately 1 to 10% of the total population of *V. parahaemolyticus* (DePaola *et al.*, 2000; Paranjypte *et al.*, 2012).

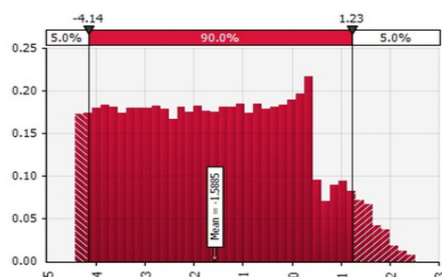


Figure 4. Simulated distribution of contamination of virulent *V. parahaemolyticus* in raw oysters at retail in log MPN/g

Outputs and risk estimate

Based on the Monte Carlo analysis, the contamination level of detectable *V. parahaemolyticus* in raw oysters at retail ranged from -2.47 log MPN/g (5% percentile) to 3.64 log MPN/g (95% percentile) and averaged at 0.37 log MPN/g (50% percentile) as shown in Figure 3. On the other hand, the contamination level of virulent *V. parahaemolyticus* in raw oysters at retail as shown in Figure 4 ranged from -4.14 log MPN/g (5% percentile) to 1.23 log MPN/g (95% percentile). The average contamination level of virulent *V. parahaemolyticus* in raw oysters at retail was estimated at -1.59 log MPN/g which was lower in comparison to the contamination level of detectable *V. parahaemolyticus* in raw oysters at retail. However, the input data might not represent a realistic picture of the oysters sold for consumption across Malaysia. The distribution of *V. parahaemolyticus* in the environment and in oysters greatly depends on geographical locations, appropriate climate and temperature, and salinity (DePaola *et al.*, 1990; Elhadi *et al.*, 2004). Hence, it is recommended that more data collection is carried out in order to roughly visualize the realistic scenario. This remains as one of the deficiencies existing in microbial risk assessment as suggested by Gardner (2004).

This model assumed that consumers will be exposed to a consumption chance of 50:50 ratio of detectable and virulent *V. parahaemolyticus* in raw oysters, which leads to adverse health effects. The assumption was made to accommodate the possibilities of consuming non-toxicogenic *V. parahaemolyticus* strains that can cause acute gastroenteritis. Report on non-toxicogenic *V. parahaemolyticus* strains with the absence of *trh* gene, *tdh* gene or urease had been found to induce fluid accumulation in suckling mice and diarrhoea in a ferret model after oral inoculation in a dose-dependent manner by Kothary *et al.* (2000). The gastroenteritis occurrence in humans caused by non-toxicogenic *V. parahaemolyticus* were reported by Garcia *et al.* (2009) and Ottaviani *et al.* (2012). This suggested that *V. parahaemolyticus* may harbour other virulence factors as the explanation

of virulence mechanism of *V. parahaemolyticus* is yet to be discovered. Based on the discovery, the focus was turned to the possibility of detectable *V. parahaemolyticus* in raw oysters to cause gastroenteritis. Admittedly, the fact that virulent *V. parahaemolyticus* remains as the main cause of gastroenteritis was accounted as well in the model. The discrete distribution simulated that the customers' dose exposure of *V. parahaemolyticus* in raw oysters was estimated at 0.0105 MPN/g for this model.

It is noteworthy that the detectable *V. parahaemolyticus* should be observed in the future microbial risk assessment of *V. parahaemolyticus* other than focusing strictly on the pathogenic (*tdh*⁺ and *trh*⁺) strains. The sudden change in the nature of *V. parahaemolyticus* had indeed increased more variability on conducting microbial risk assessment for this pathogen as previous researchers (Yamamoto *et al.*, 2008; Norrakiah *et al.*, 2013) were mainly attentive towards the pathogenic strains.

The dose-response model predicted that the probability of illness per serving of the consumers was 4.83×10^{-9} . When multiplied with the assumed 365 servings per year, the probability of illness annually was 1.76×10^{-6} . Based on the Malaysian population to date obtained from Department of Statistics, Malaysia (2013), the total population was 29,947,600 people. By referring to the data provided by MOH, Malaysia (2003) of the habitual food consumption, it was estimated that approximately 58.81% of the population consumed shellfish which translates to a total of 17,612,184 people. The expected number of annual cases was 31 cases with a rate of 0.104 per 100,000 people of *V. parahaemolyticus* infection from raw oyster consumption based on this model.

As the microbial risk assessment was conducted, a number of limitations were identified along the process. The suggested model did not include the growth model of *V. parahaemolyticus* during the time gap from hypermarkets to consumption. This had resulted in a lack of quantitative data which has indirectly underestimated the infection frequency of *V. parahaemolyticus* in raw oysters. However, the model's assumption can be referred for food retail outlets that serve fresh raw oysters where there is minimal time for the growth of *V. parahaemolyticus* to occur. In addition, the predicted cases did not include the possibility of one getting infected by other opportunistic human pathogens such as other vibrios, *Aeromonas* spp., and *Pseudomonas* spp., that can cross-contaminate during harvest to the consumption stage in oysters. The compounding effect of these microbes is likely to weaken the human immune system, which leads to disease that may not be caused

by the dose of a single organism (Dickinson *et al.*, 2013). On the other hand, the beta-Poisson model assessed was conducted based on healthy individuals. Data of at risk groups who are children, elderly, and immunocompromised individuals, especially those with liver disease are still lacking in predicting the actual risk of the pathogen. The possibility of these risk groups to be infected severely is far greater than healthy individuals. The severity of the gastroenteritis caused by *V. parahaemolyticus* depends greatly on the dose of bacteria consumed and virulence degree of isolates (DePaola *et al.*, 1990). It also depends on the person's immune susceptibility. Thus, the assessment might have underestimated the expected cases. On the other hand, the data of consumption pattern was not solely on oysters, but shellfish as a whole recorded by MOH Malaysia (2003). Therefore, the prediction of cases could have been overestimated as raw oysters are consumed by a group of minorities in Malaysia due to the costly price of raw oysters.

Although the health effects caused by *V. parahaemolyticus* are not severe, short in duration and symptoms are self-limiting as characterized in the hazard characterization, reduction of the risk of being infected by this pathogen is recommended with the aim to provide better safety consumption of seafood, particularly raw oysters. Significantly, understanding the ecosystem of *V. parahaemolyticus* is paramount to devise methods of control (Beuchat, 2002). The risk reduction can be carried out by closely monitoring the initial level of the pathogen, reducing the contamination level of the pathogen and preventing an increase in the number of the pathogen. Firstly, places and times of oyster collection should be ensured to have minimal to zero contamination of *V. parahaemolyticus*. Consequently, this will control the initial level of *V. parahaemolyticus* present in oysters and further carrying out methods that prevent contamination of oysters during harvesting and transport. On the one hand, if the potential risk remains uncertain and collection was carried out, post-harvest processing using procedures proven to reduce risk to tolerable levels should be performed. For oysters, shucking using high pressure processing or sterilization can be done during the post-harvest processing. These methods have been proven to reduce the contamination level to a minimum, but at the same time incur some changes to their taste and texture (Rees *et al.*, 2010).

Enforcement of Good Hygiene Practice (GHP), Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Point (HACCP) at food industries, hypermarkets and food retail outlets are essential to minimize the survival chances of

pathogen and control its growth. For the consumption of raw oysters, the programs are mandatory in order to reduce the infection levels of pathogens to minimal with strict surveillance. However, the sustainability of the enforcement remains a challenge to the food safety world. The effectiveness in practice has been always at a question mark. Local authorities should fulfil their role of ensuring the compliance and evaluate the regulations at all times.

Besides that, public education on basic food safety should be constantly conducted by the local government. The public should be educated that foodborne illness must never be measured as a minor illness and they should seek early treatment. It is recommended that the local government strengthens the dialogue with the public to channel any information on the hazards associated with foods and the relevant control measures. Constant integrated monitoring and surveillance should be conducted for immediate detection of foodborne pathogens. This eventually helps minimizing the risk of pathogen exposure to the public and, for early identification of emerging problems and changing trends. Epidemiological surveillance in humans and animals is also another critical component of public health in which it provides early resource planning and measuring the impact of control strategies. More importantly, it allows preparations to respond to emerging risks.

Rapid sharing of information and data to protect food safety and public health around the globe is recommended through constant updates on researches at both national and international levels. The information serves as a source to conduct ideal models for microbial risk assessment. Microbial risk assessment remains as one of the tools that can be used to predict the risk estimate and provides inferences as well as a range of mitigations for risk managers to take to improve public health (FAO/WHO, 2011). It is also considered as a proactive action to determine the effectiveness of intervention measures throughout the whole food chain, or combinations of intervention measures, on public health (Havelaar *et al.*, 2004).

Antibiogram of V. parahaemolyticus isolated from raw oysters

The possibility of mutation in *V. parahaemolyticus* against antibiotics should not be ignored. Emerging antibiotic resistance strains of *V. parahaemolyticus* had been foreseen as a serious threat in seafood industries. The public's health is also at stake as the resistance genes might spread to them, or degrade the efficacy of the current antibiotics, which can exacerbate the problem of removing the pathogens and increase the severity of infection.

Table 4. Antibiotic Resistance Pattern and the MAR Index of the *V. parahaemolyticus* isolates

Isolates	Antibiotic Resistance Pattern ^a	MAR Index
VP10	B Va	0.17
VP18	B Va	0.17
VP11	B Va	0.17
VP9	B Va	0.17
VP23	B Va	0.17
VP16	B Va S	0.25
VP22	B	0.25
VP21	B	0.25
VP20	B	0.25
VP1	B Va S	0.25
VP6	B Clr Nor Va	0.33
VP12	B S	0.33
VP7	B Clr E Va	0.33
VP8	B Clr E Va	0.33
VP15	B Clr S Va	0.33
VP2	B Clr E Va	0.33
VP3	B Clr E Va	0.33
VP14	B Clr E S Va	0.42
VP17	B Clr E S Va	0.42
VP19	B Clr E S Va	0.42
VP13	B Clr E S Va	0.42
VP4	B C Clr	0.42
VP5	B C Clr E S Va	0.50

^a List of Antibiotic used: Imipenem (IPM), Norfloxacin (NOR), Erythromycin (E), Bacitracin (B), Kanamycin (K), Gentamicin (GM), Chloramphenicol (C), Clarithromycin (CLR), Amikacin (AN), Vancomycin (VA), Nalidixic Acid (NA), Streptomycin (S).

Figure 5 shows the antibiotic resistance profile of *V. parahaemolyticus* isolates based on the different types of antibiotics tested. The 23 isolates of *V. parahaemolyticus* isolated from raw oysters showed high resistance against Bacitracin (100.00%) and Vancomycin (100.00%) followed by Clarithromycin (65.22%), Erythromycin (52.17%), and Streptomycin (43.48%) which showed intermediate resistance. Only 8.70% of the isolates showed resistance towards Chloramphenicol. The resistance pattern was recorded as follows: Bacitracin>Vancomycin>Clarithromycin>Erythromycin>Streptomycin>Chloramphenicol.

The high resistance of the isolates towards Bacitracin and Vancomycin was mainly due to the mechanism of these antibiotics, targeting the bacterial cell wall that is usually effective in Gram-positive bacteria. As *V. parahaemolyticus* is a Gram-negative bacterium, the mechanism of action of these antibiotics is ineffective. The results were supported by the findings of Shantini *et al.* (2004) and Wong *et al.* (2000) that most environmental isolates showed similar resistance towards these antibiotics. The rate of resistance towards Chloramphenicol, Rifampicin and Doxycycline among *V. parahaemolyticus* isolates is becoming a concern as reported by Rahman *et al.* (2008). This is in agreement with the results of the present study where there was evidence related to the resistance of Chloramphenicol. One of the possibilities of the resistance was the high dosage of Chloramphenicol used, mostly in aquaculture farms. The selection pressure from the antibiotic used induced *V. parahaemolyticus* strains to become adaptive towards the antibiotic doses. This increases

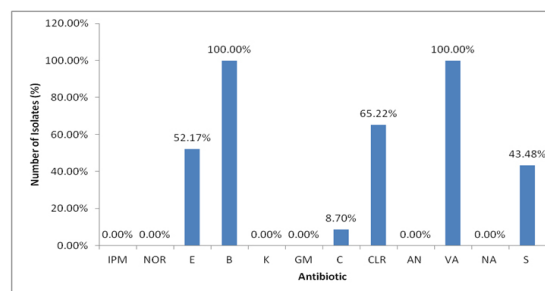


Figure 5. Antibiotic resistance profile of *V. parahaemolyticus* isolates based on the 12 types of antibiotics tested. List of Antibiotic used: Imipenem (IPM), Norfloxacin (NOR), Erythromycin (E), Bacitracin (B), Kanamycin (K), Gentamicin (GM), Chloramphenicol (C), Clarithromycin (CLR), Amikacin (AN), Vancomycin (VA), Nalidixic Acid (NA), Streptomycin (S).

its survival and, at the same time, the strains will develop to be more pathogenic (Beuchat, 2002; Laximinarayan *et al.*, 2013).

All isolates were susceptible to a total of six of the antibiotics tested, namely Imipenem, Norfloxacin, Kanamycin, Gentamicin, Amikacin, and Nalidixic Acid, indicating that these antibiotics are still active against *V. parahaemolyticus*. It should be highlighted here that the data demonstrated for Nalidixic Acid were similar to Manjusha *et al.* (2005), Zulkifli *et al.* (2009) and Yu *et al.* (2013) but contradicting with that of Tunung *et al.* (2012) on *V. parahaemolyticus* contamination of vegetables. Likewise, Wong *et al.* (2012) reported a resistance pattern for Amikacin in *V. parahaemolyticus* isolates from shrimps which was in conflict with the results recorded in this study. It is suggested that the different antibiotic resistance patterns of *V. parahaemolyticus* was built up through environmental factors which largely influenced the genetic factors of the microorganism.

The MAR Index of *V. parahaemolyticus* isolates ranged from 0.17 to 0.50 as shown in Table 4. All isolates showed multiple antibiotic resistance up to more than two antibiotics with 78.26% of the isolates exhibiting a MAR index of more than 0.2. Of the total 23 isolates, 7 isolates (30.43%) were resistant to four types of antibiotics while only 1 isolate (4.35%) was resistant to six out of the twelve types of antibiotics tested. The remaining isolates were equally divided among two, three and five antibiotics, where each had 5 isolates (21.74%) being resistant, respectively.

The main suggested factor contributing to the antibiotic resistance of *V. parahaemolyticus* is the mutation of the cellular DNA that modified the antibiotic target site or transport mechanisms which caused a decrease in the action of the antibiotic towards the cell (Zulkifli *et al.*, 2009). Another possible factor could be the inability of the antibiotic to enter the bacterial cell and reach its target site.

Furthermore, the possibility of the microorganism acquiring resistance through the transmissible nature from other microorganisms should be accounted as well either through intra or interspecies (Romero, 2012).

Bacteria showing resistance to more than three antibiotics become a call for serious concern, especially pathogenic bacteria like *V. parahaemolyticus* (Romero, 2012). It is no doubt that researchers should be continuously alert on this concern if there is a possibility of development of bacteria that become resistant towards most of the antibiotics used which eventually increase the risk of infection unknowingly. Aforementioned that several researchers had reported different resistance patterns of *V. parahaemolyticus*, there is a rising possibility of a multidrug resistant *V. parahaemolyticus* which becomes a gap of knowledge to be filled for public health concern. The different resistance patterns of *V. parahaemolyticus* also signify the lack of efficacious antibiotics. Thus, it has indirectly increased the exposure of the public to the pathogens and putting the public's health at risk. Nonetheless, it is recommended that more studies should be conducted on multidrug-resistant *V. parahaemolyticus* in order to control the progress of the emergence of multidrug resistant *V. parahaemolyticus* (Wong et al., 2012).

The antibiotic resistance of *V. parahaemolyticus* can also be closely related to its ability to form biofilm. Aforementioned, biofilm provides a protective environment for the pathogens, where it reduces the effectiveness of inhibitory agents such as antibiotics, detergents and sanitizers (Morris et al., 1997). Therefore, the attachment of *V. parahaemolyticus* in biofilm may enhance their survival and growth as it is resistant against antibiotics, which will increase the probability of cross-contamination and infection.

The issue of antibiotic resistance spread sparks the need for global solutions with rising evidence of the potential effect of the spread of antibiotic resistance bacteria and resistance genes. Exposure through food is the most important commonly studied transmission route. As food is traded globally and international travel, it can lead to local food security problems with negative effects on public health. The agreement with the Codex Alimentarius Commission (CAC) on the guidance of risk analysis of foodborne antimicrobial resistance had initiated as part of the solution to this issue. However, large amounts of data, including relevant endpoints, such as public health burden of resistance is required. To add on to the complications, the different possible transmission routes and attempts of estimating the risk in various ways had consequently yielded a wide range of

results. Despite that, the antibiotic resistance data on *V. parahaemolyticus* collected here can serve as a reference for future attempts of risk assessment of foodborne antimicrobial resistance (Laximinarayan et al., 2013).

Conclusion

The prevalence of 50.00% *V. parahaemolyticus* enumerated using MPN-PCR method in raw oysters with a prediction of 1.76×10^{-6} yearly of probability illness indicated that consumption of raw oysters is a risk factor for *V. parahaemolyticus* infection. The inevitability of the prevalence of *V. parahaemolyticus* in seafood has become a concern to the public health as this pathogen is known as the frequent causal agent of gastroenteritis in Asia. It is noteworthy to have rapid detection methods of foodborne pathogens for time and labour saving with reliable results. Significantly, these rapid detection methods have become one of the most suggested tools in controlling foodborne illness. Currently, rapid detection methods are coupled with quantitative microbiology has become a fertile basis for microbial risk assessment in order to conduct a more extensive analysis of the risk factors. The availability of the scientific data had enabled microbial risk assessment to become an invaluable tool in predicting the risk estimate and propose further actions to reduce microbial risks for public health improvement. The data collection of antibiotic characterization of *V. parahaemolyticus* can be further used for risk profiling of *V. parahaemolyticus* in the future that will serve as an advantage in risk assessment. Although the microbial risk assessment conducted in this study had several limitations, it can serve as an eye opener to researchers for further improvement to develop a more refined microbial risk assessment of *V. parahaemolyticus*.

Acknowledgements

Research fund was sponsored by E-Science Fund from the Ministry of Science, Technology and Innovation, Malaysia and in part by Kakenhi Grant-in-Aid for Scientific Research (KAKENHI 24249038), Japan Society for the Promotion of Sciences and grant-in-aid of Ministry of Health, Labour and Welfare, Japan.

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