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Enumeration and Survivability Assessment of Three Major *Vibrios* in Frozen Sushi using Multiplex MPN-PCR Assay

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Abstract

Sushi is one of the popular and widely consumed Japanese cuisines in Malaysia. *Vibrio vulnificus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* are the most deleterious fish borne pathogens for humans. Although the occurrence of few other bacterial pathogens has been tested in sushi, the presence and survivability pattern of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* in Ready-to-Eat (RTE) sushi in Malaysia have not been investigated thoroughly. Therefore, the possible threat to food safety associated with the consumption of contaminated sushi remains poorly detected. We addressed this research gap by enumerating *V. vulnificus, V. cholerae* and *V. parahaemolyticus* in RTE sushi using a low-cost multiplex Most probable number-PCR (MPN-PCR) system. The MPN-PCR counts were studied and the viability pattern of the target bacteria under three different storage conditions was presented. A total of 70 sushi samples were analyzed and 16 (22.86%), 8 (11.43 %) and 4 (5.71%) samples were found to be positive for *V. parahaemolyticus, V. vulnificus* and *V. cholerae,* respectively.

Keywords: Food-borne pathogen; Multiplex MPN-PCR; Sushi*; Vibrio vulnificus*; *Vibrio cholerae*; *Vibrio parahaemolyticus*

Introduction

Sushi is one of the most popular and widely consumed Japanese cuisines and is prepared with cold cooked rice by acidifying with vinegar. It is made into bite-sized pieces with different toppings including raw or undercooked fish or seafood [1]. The distinct feature of sushi is the advantage of direct consumption of fresh raw fish or seafood without further cooking [2]. However, the Australia-New Zealand Food Standards Code labels "sushi" as a potentially hazardous food because raw fish is known to carry diverse pathogenic microorganisms and so consumption of raw fish could pose potential health risk [1]. Moreover, the preparation process of sushi involves minimal heat treatment and direct hand contact of the chefs and thus increases the likelihoods of contamination [3]. Although maintaining the low temperature of food storage has been considered to reduce the possibility of bacterial contamination, several occurrences including *Salmonella* outbreaks of Singapore [4], *Escherichia coli* outbreaks of Nevada [5], *Salmonella* outbreaks of Australia [6] have been found to be associated with sushi consumption. Likewise, several studies have reported the association of consumption of sushi with the infection of bacteria [7-10] including *V. parahaemolyticus* [11] and *V. cholerae* [12].

The abundance of *Vibrio* species in raw fish and marine products implies that these are potential reservoirs [13]. Among the pathogenic species, *V. vulnificus, V. cholerae* and *V. parahaemolyticus* are the most commonly encountered in raw fish [14,15]. Many Asian countries, including Japan, China, and Taiwan consider *V. parahaemolyticus* as a common cause of foodborne illnesses whereas in the United States it is recognized as the principal cause of human gastroenteritis related to seafood consumption [16]. Transmission of *V. cholerae,* a causative agent of cholera, occurs from their aquatic niches to humans via seafood or various contaminated foods or water [17]. *V. vulnificus* has been reported to cause primary septicemia in humans with a high fatality rate (50-60%) due to consumption of raw or undercooked seafood like raw oysters [18]. Consumption of food contaminated with these *Vibrio* species can cause a large number of other diseases that include but not limited to traveller's cholera, diarrhea, bloody diarrhea, nausea, vomiting, wound infection, abdominal pain,

severe gastroenteritis and fever in healthy and immune-suppressed people [19].

It is recommended that freezing and storing seafood at -20 ˚C (-4 ˚F) or below for 7 days with subsequent refrigeration (below 5 ˚C) for a maximum of 12 h could prevent the bacterial contamination. However, serving and consumption should be within 2-4 hrs once taken out from the sustained temperature [4,20]. Nevertheless, the efficacy of deep-freezing in preventing microorganisms depends on the interrelationship between various factors including the freezing temperature, time duration needed to freeze the tissues, the fish fat content, the type of bacteria and the density of the bacteria [14].

However, despite having pressing needs, no study to date has examined the occurrence and the viability pattern of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* in RTE sushi in Malaysia. For the first time, we evaluated 70 sushi samples under three different sets of conditions, namely, the original state, 4-5 h storage at room temperature, and 7 days of freezing, as practiced in sushi catering service worldwide, and then established the survivability pattern of these target *vibrios* in sushi.

The conventional culture-based methods for detection of microorganisms are non-specific, time-consuming, and laborious [21,22]. Therefore, rapid and easy detection is a present-day demand. In recent years, several simplex PCR [23,24] and multiplex PCR [25-27] methods have been reported for the detection of several *Vibrio* species. However, none of these techniques could estimate their concentration as well as could discriminate the presence of viable and dead cells. Various MPN-PCR techniques have been documented to identify *V. parahaemolyticus* [28,29], *V. cholera* [30,31], and *V. vulnificus* [31,32].

The Most Probable Number (MPN) method was introduced to identify and quantify the microbial load effectively based on probability [33]. This technique is especially applied for detecting low-level microbial contamination as well as enumerating the live cells [34]. Nowadays, multiplex MPN-PCR assay has got huge attention due to its multi-target detecting capability in a single assay platform, curtailing identification time and cost [35]. Recently, we have documented a multiplex MPN-PCR assay able to enumerate the live cells of these three target *vibrios* in a single reaction tube [33]. The present study incorporates the reported multiplex MPN-PCR method for enumerating *vibrios* in frozen sushi and to assess their viability pattern. To the best of our knowledge, this is the first report on multiplex MPN-PCR counts to provide an insight into the pattern of occurrence of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* in frozen sushi sold in Malaysian supermarkets.

Materials and Methods

Sample collection

Different types of Ready-To-Eat (RTE) frozen sushi (n=70) were purchased from various supermarkets in Selangor and Kuala Lumpur, Malaysia between 11:00 am and 2:00 pm in 10 days (Table 1). All the samples were kept in the stomacher bag and placed into insulation box with ice packs for carrying to the laboratory. The samples were transported from the market to the laboratory within 1 h and then processed for subsequent analysis. Each sample was assigned to an identification code to maintain a database of the isolates and equally divided in three portions for subsequent analyses.

No.	Types of Sushi	ID	Number of Sushi
1	Fresh Salmon	FS1-10	10
2	Aburi Salmon	AS1-10	10
3	Jelly Fish with Chinese Sauce	JFCS1-10	10
4	Inari Tuna	IT1-10	10
5	Inari Kani Mayo	IKM1-10	10
6	Black Ebiko	BE1-10	10
7	Chuka Tako	CT1-10	10
	Total		70

Table 1: List of sushi samples.

Sample preparation

Each sample was equally divided into three portions inside the laminar flow using foil paper for subsequent analysis with proper care avoiding chances of contamination. The first portion of the sushi samples was analyzed within the first hour of processing to check the microbial load at the point of sale using the optimized multiplex MPN-PCR method. The second portion was stored at room temperature for 4-5 hrs and the bacterial growth was assessed under normal environment in order to monitor any increase in microbial load without refrigeration after few hours of sushi purchase. The third portion of samples was stored frozen at -20 ^{0}Cfor 7 days and microbial load was measured subsequently in order to observe if the recommended 7 days of freezing could prevent the microbial growth and ensure the food safety of sushi [4].

Preparation of controls

Positive controls were prepared according to a method established by Teh et al. [36] with some minor modifications. Briefly, a single colony of each *V. vulnificus, V. cholerae* and *V. parahaemolyticus* was inoculated in 1 mL of Luria Bertani (LB) broth, supplemented with 3.0% NaCl, and incubated overnight at 37 ˚C. An aliquot of 100 µl from each of the overnight cultures of the three *vibrios* was spiked with 5 g of the fresh sushi sample and was incubated at room temperature for 30 minutes. After homogenization with 45 mL of the Alkaline Peptone Water

(APW), the spiked sample was incubated overnight at 37 ˚C. A 10-fold serial dilution (10^{-1} -10^{-9}) was carried out. An aliquot of 100 µl of each diluted portion was boiled, centrifuged and the resultant supernatant was used as DNA template for direct PCR analysis. On the other hand, a pair of primers (used from previously published literatures) which amplified 187 bp site from pQE-30 plasmid were used as an Internal Amplification Control (IAC) [37]. Sterile deionized water was used as the template in the PCR negative control.

Enrichment

In this study, a two-step enrichment procedure that has been evaluated to be more efficient than one step enrichment [38], was applied. In the first step, 10 g of each sample was added to 90 ml of Tryptic Soy Broth (TSB; BactoTM, France) supplemented with 3% sodium chloride (NaCl; Merck, Germany) and the mixture was homogenized followed by pre-enrichment through incubation at 37 ˚C for 6 h before further analysis was done. In the next step, the dilution of the pre-enriched samples was done from 10^{-1} to 10^{-7} in Salt Polymyxin Broth (SPB; Nissui, Japan) followed by incubation of the tubes at 37 ˚C for 14 to 16 h. Following enrichment, PCR assay was carried out with diluted samples which were found turbid and the concentrations (MPN g^{-1}) of viable microorganisms present in the raw fish samples were determined from the calculation applying computer-assisted Microbiological Methods & Bacteriological Analytical Manual (BAM) [39].

DNA extraction

The boiled cell method [40] was applied for the extraction of DNA from all turbid tubes. Herein, some modification of the boiled cell method was made to eliminate any inhibitory effect in subsequent PCR analysis. Briefly, 1 ml of clear food homogenate was taken in a 1.5 ml microfuge tube and then centrifuged at 12,000 rpm for 3 min. After discarding the supernatant, the pellet was re-suspended in 100 µL of 1X PBS buffer of pH: 7.4 and centrifugation was carried out again at 12,000 rpm for 2 min. This washing step with 1X PBS buffer was repeated once and the supernatant was discarded. A 100 µL of sterile distilled water was added to the pellet, boiled for 10 min and snapped cooled in ice. Then, the cell suspension was re-centrifuged at 13,400 rpm for 3 min. Approximately 80 µL of supernatant containing the crude DNA was transferred to sterile micro-centrifuge tube and preserved at -20 ˚C for future use.

Multiplex PCR

In this study, species-specific primers were used from previously published literatures. The sequences of the primers for identifying the *vibrio* species are given below: For *V. vulnificus,* Forward: 5′-TTCCAACTTCAAACCGAACTATGAC-3′; Reverse: 5′-ATTCCAGTCGATGCGAATACGTTG-3′ [41], for *V. cholerae,* Forward: 5′-CAGTAAAGAAACGACCAAACTC-3′; Reverse: 5′-TGCCAGTTTCGATGATGCCG-3′ [42] and for *V. parahaemolyticus,* Forward: 5′-AGCAAGTTTCGATGATGCTG-3′; Reverse: 5′-ACCAGCAACCAAAACTTTCGCT-3′−[42]. The primers for *V. cholerae* and *V. parahaemolyticus* amplified 338 bp and 409 bp PCR products from *pnt*A gene whereas those for *V. vulnificus* amplified 205 bp amplicon from *vvh*A gene. The multiplex PCR assay was carried out in a total of 25 µL reaction volume comprising of 5 µL of 5× PCR buffer, 1.0 µL of 10 µM of each primer, 1.0 µL of 10 mM dNTP (deoxynucleoside triphosphate) mix, 3.0 µL of 25 mM $MgCl_2$, 0.125 µL of *Taq* polymerase (5U/µL), 2.0 µL of DNA template (50 ng/ µL) and 2.0 µL of IAC template and required amount of ddH_2O. A negative control was made by replacing template DNA with deionized water. The cycling parameters consisted of initial denaturation at 94 ˚C for 4 min followed by 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 60 ˚C for 1 min, extension at 72 ˚C for 1 min and the final extension at 72 ˚C for 5 min. For separation, the PCR products were loaded in 2% agarose gel stained with Florosafe DNA stain (First Base, Selangor, Malaysia) followed by electrophoresis at 120 V for 80 min in 1 × TBE (Tris borate-EDTA) buffer. The gel was visualized using a Gel Documentation system (AlphaImager HP, Alpha Innotech Corp., California, USA). A 50 bp molecular size marker (Promega, USA) was used to assess the fragment length. All experiments were performed in three different trails and the mean value was chosen for the result. All the PCR analyses were repeated twice.

DNA sequencing

Purification of PCR products was performed using a PCR purification kit (First Base, Selangor, Malaysia) followed by visualization of the samples in 1.5% agarose gel by applying about 1 µl purified samples. The purified PCR products were sequenced bi-directionally in an ABI PRISM 96-capillary 3730xl genetic analyzer (Applied Biosystems, USA) using BigDye® Terminator v3.1 cycle sequencing kit [43].

MPN analysis

MPN counts (MPN g^{-1}) were calculated based on the Microbiological Methods & Bacteriological Analytical Manual (BAM) [39] to monitor the occurrence of *V. vulnificus, V. cholerae*, and *V. parahaemolyticus* in frozen sushi samples.

Results and Discussion

Commercially prepared RTE sushi products available in Malaysian supermarkets were targeted because raw sushi prepared in restaurants or at home are consumed within a short time of preparation, unlike the commercially prepared RTE frozen sushi. The time and temperature profiles during preparation and storage of industrially prepared sushi until consumption are critical with respect to both shelf life and food safety [44].

Detection of *vibrios* using multiplex PCR

Multiplex PCR for *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* along with IAC was optimized for the sushi samples following our previous work [33]. For the detection of *V. cholerae* and *V. parahaemolyticus,* the house-keeping gene *pntA* coding for a transcription factor regulator was utilized. While, *vvhA* gene, responsible for coding for a transmembrane transcription activator for hemolysis and cytolysis were used to detect *V. vulnificus*. Through the use of an IAC, a non-competitive internal amplification control, the reactions indicating a negative PCR result could easily be interpreted as there was a constant generation of a control signal by the IAC when there were no other targets [45]. To run as positive control, spiked samples having reference strains of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* were used in all stages of the assay in order to eliminate the anticipation of any false negative result. On the other hand, a negative control was used to avoid anticipation of any false positive result. Sequencing of species-specific amplicons for *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* revealed 99% similarities with the respective reference strains and this validates successful amplification of DNA fragments. Furthermore, the MPN-PCR outcomes reveal the presence of *vibrios* in sushi at the selling point and display the viability pattern of those bacteria surviving after 4 - 5 h and 7-days of freezing (Figure 1).

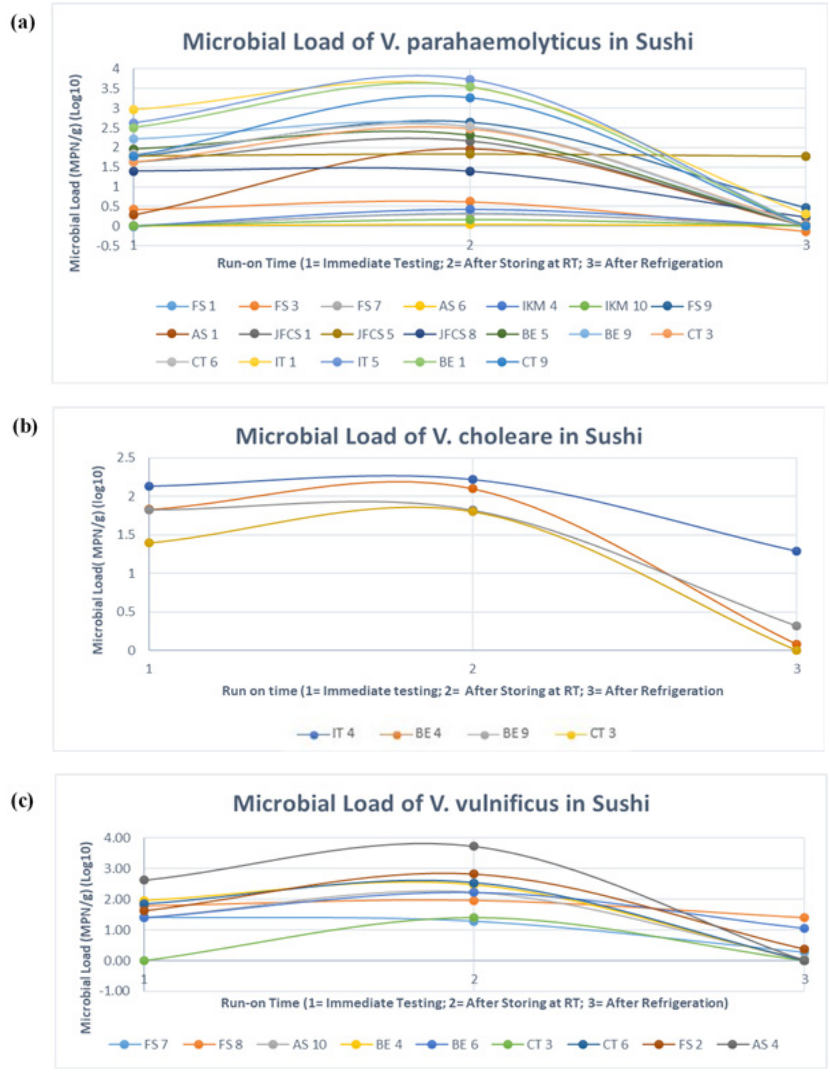

Figure 1: Distribution of MPN-PCR count of *V. parahaemolyticus* (a), *V. cholerae* (b), *V. vulnificus* (c) in refrigerated sushi obtained by Multiplex MPN-PCR. Run-on time (X-axis) "1" stands for the initial state of sell while "2" and "3" represent the states after 4-5 h and 7 days of freezing, respectively. The mean values of all three trails are used.

Enumeration of target *vibrios* in sushi

Out of 70 sushi samples, 16 (22.86%), 8 (11.43 %) and 4 (5.71%) samples were found to be positive for *V. parahaemolyticus, V. vulnificus* and *V. cholerae,* respectively, at the selling point. Following 4-5 h of storage at room temperature (25-27 ^{0}C), 20 (28.57%), 9 (12.86%) and 4 (5.71%) samples were found positive for *V. parahaemolyticus, V. vulnificus* and *V. cholerae,* respectively. Furthermore, after 7 days of freezing at -20 ^{0}C, 7 (10%), 6 (8.57%) and 3 (4.29%) samples were found to contain *V. parahaemolyticus, V. vulnificus* and *V. cholerae,* respectively (Table 2 and Figure 2). The microbial loads of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* were determined in positive samples in three different storage conditions. The concentrations of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* were found 9.79 x 10^{-1} - 9.17 x 10^2, 2.49 x 10^1 - 1.36 x 10^2 MPNg^{-1} and 2.49 x 10^1 - 4.22 x 10^2 MPN g^{-1}, respectively, in the initial state of sell while those were 1.12 x 10^0 - 5.36 x 10^3, 6.36 x 10^1 - 1.66 x 10^2 MPNg^{-1} and 1.95 x 10^1 - 5.36 x 10^3 MPN g^{-1} respectively following 4 - 5 h of storage and 7.36 x 10^{-1} - 5.88x10^1, 1.22 x 10^0 - 1.95 x 10^1 MPNg^{-1} and 9.79 x 10^{-1} - 2.49 x 10^1 MPN g^{-1}, respectively after 7 days of freezing. A sharp rise in the microbial loads (MPN g^{-1}) was observed after leaving the sushi for 4-5 h at room temperature while the bacterial concentrations declined after 7-days of freezing period, although the sushi samples were not found entirely pathogen free.

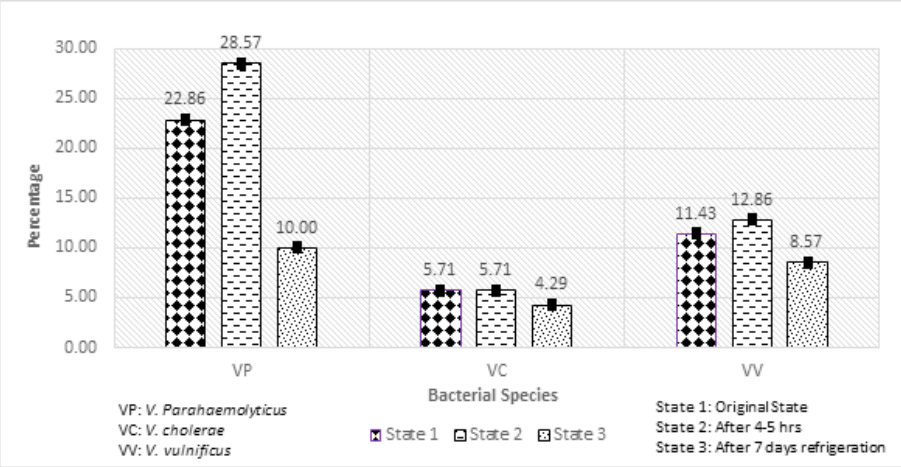

Figure 2: Percentage of the target *vibrios* in the sushi samples in three different storage conditions.

Species	n	Original State						Following 4-5 hrs. storage						Following 7 days Refrigeration					
		Trail 1		Trail 2		Trail 3		Trail 1		Trail 2		Trail 3		Trail 1		Trail 2		Trail 3	
		+	%	+	%	+	%	+	%	+	%	+	%	+	%	+	%	+	%
VP	70	16	22.8	16	22.9	16	22.9	20	28.6	20	28.6	20	28.6	7	10	7	10	7	10
VC		4	5.7	4	5.7	4	5.7	4	5.7	4	5.7	4	5.7	3	4.3	3	4.3	3	4.3
VV		8	11.4	8	11.4	8	11.4	9	12.9	9	12.9	9	12.9	5	7.1	5	7.1	5	7.1

Table 2: Occurrence of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in RTE refrigerated sushi in three different conditions.

Survivability of target *vibrios* in sushi in three different storage conditions

The variation in time and storage conditions was assessed to evaluate the changes in bacterial growth upon storage at room temperature and the recommended 7-days of freezing period. The distribution of MPN-PCR count of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* in refrigerated sushi obtained by MPN-PCR is shown in Figure 1 (A-C). The MPN-PCR values (MPN/g) were placed over several ranges (Y-axis) in terms of log10 and the run-on time (X-axis) of each of the *vibrios* was calculated. It can be speculated from the plot that the microbial load was increased after leaving the sushi for 4-5 h at room temperature which is optimal for bacterial growth and it posed an increased health risk to the consumers. The recommended 7-days freezing period [46] reduced the bacterial growth but it could not make the sushi entirely pathogen free.

The survivability patterns of target bacteria in the analyzed sushi samples indicate that the transmission of these bacteria could occur from their aquatic niche to humans either through the ingredients used to prepare the food or through supply chain cross-contamination [47]. Factors such as handling, processing and display along with the storage condition may influence the microbiological safety of these RTE food [48]. *Vibrio* can multiply rapidly in elevated temperatures [16] and survivability assessment of these target bacteria in three

different conditions should be considered to ensure the food safety. The first portions were analyzed in the first hour for determining the occurrence of the target bacteria in the original state of the sushi. According to Borresen [49], doubling time of *vibrios* under ideal conditions can be less than 10 min. Therefore, the second portions of the sushi samples were stored in room temperature (26-27 ˚C) for 4 -5 h to determine their microbial load. This is to simulate the situation when a consumer consumes the food after several hours of buying without maintaining temperature below 5 ˚C. Factors including handling, processing and display along with storage might have an influence on the microbiological load of these ready-to-eat foods at the point of sale or consumption [48]. Considerable augmentation in microbial load was recorded in this phase of the study revealing a potential health risk to the consumers. The primary control measure for *Vibrio* is to prevent multiplication of the organism. Hence, freezing and storing seafood at -20 ˚C (-4 ˚F) or below for 7 days is recommended to avoid the rise in microbial load [46]. Therefore, the other portions of the samples were frozen prior to the analysis following the recommendations and a drastic decline of microbial loads was recorded in the tested sushi samples. The result showed that freezing significantly reduced the microbial growth but failed to make the sushi totally free of pathogens. According to Randa et al. [50], in winter season, *V. vulnificus* occurs in low number because the bacteria stay at viable but non-culturable (VBNC) state at low temperature and the microbial presence in the sushi samples even after 7 days of freezing could be associated with this survival strategy of bacteria. Moreover, the use of MPN-PCR assay instead of the cultural method or conventional PCR approaches facilitated the detection of VBNC bacteria through the two-stage enrichment approach that resuscitates the sub lethally injured bacterial cells and permitted the determination of bacterial presence under all stages of bacterial growth, unlike the conventional culture-based method [33].

Previously, Hara-Kud et al. [51], reported a comparison-based study between MPN-PCR method and MPN-culture method to enumerate *V. vulnificus* in seafood and found that MPN-PCR showed higher sensitivity than MPN-culture based method. Many studies have reported the presence of different bacteria in sushi. Puah et al. [10], reported the presence of 26% *Staphylococcus aureus*, and 16% *Salmonella enterica* in 200 sushi and sashimi samples in Malaysia based on their phenotypic profiles and PCR analysis. Atanassova et al. [7] compared the microbial quality of the freshly prepared sushi from sushi bars and RTE frozen sushi from supermarkets and found that the industrially prepared sushi has better microbiological quality than that of freshly prepared sushi. In another study, Liang et al. [52], demonstrated the microbiological quality of take-away raw salmon finger sushi sold in Hong Kong and suggested that improper handling of ready-to-eat foods may lead to outbreaks of food poisoning. Jain et al. [5], reported a disease outbreak associated with sushi restaurants in Nevada in 2004 due to the possible presence of enterotoxigenic *Escherichia coli*. Besides, Suppin et al. [53], found *Yersinia* sp. and *Listeria* sp. in 5% and 3% of samples, respectively from the sushi samples in Vienna. Likewise, 53 people in nine states of USA have been reported sickened with *Salmonella* Paratyphi B in an outbreak after consumption of sushi with raw tuna. This outbreak was linked with frozen, pre-packaged raw tuna indicating that freezing might slow down the growth of *Salmonella* but cannot ensure the killing of bacteria [54]. Therefore, the microbiological quality of the sushi was found questionable as reported in previously published articles and our findings also strongly support this.

The current study quantified the occurrence of the three major *vibrios* that was performed in single assay platform combining PCR and MPN methods and revealed their viability pattern in three different conditions. Moreover, this study attempts to demonstrate the importance of handling precautions during the preparation and subsequent storage of RTE food in Malaysia. It also highlights the significance of implementing Food Safety Management Systems based on Hazard Analysis and Critical Control Point to ensure safe intake of the popular food sushi.

Conclusion

Contamination of sushi with *Vibrio* species has direct impact on public health and the occurrence of *V. vulnificus, V. cholerae* and *V. parahaemolyticus* in RTE Malaysian sushi reflects that they can be an imperative source of food-borne illness if not prepared in a hygienic condition and stored properly. The integrated approach by incorporating MPN techniques in multiplex PCR assay is highly promising with enhanced efficiency for quantitative detection of multiple species in a single assay platform. The present findings highlight the magnitude of public health risk associated with *V. vulnificus, V. cholerae* and *V. parahaemolyticus* borne diseases attributed to routine intake of raw fish carrying these bacteria. Moreover, the survivability pattern demonstrates that the recommended freezing cannot prevent the bacterial growth entirely. The increased microbial load of the *vibrios* in sushi after 4-5 h of storage at room temperature clearly indicates the possibility of health risk if consumed in that state. At least 7 days freezing at -20 ^{0}C with subsequent storage at below 5 ^{0}C till serving could control the bacterial growth. Therefore, freezing and storing seafood at -20 ^{0}C or below for 7 days, as recommended to avoid the rise in microbial load, should be strictly maintained and monitored to ensure that sushi is safe for consumption.

Conflict of interest

All authors declare that there is no conflict of interest to publish the content of this article.

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References

1. Nawa Y, Hatz C, Blum J (2005) Sushi delights and parasites: the risk of fishborne and foodborne parasitic zoonoses in Asia. Clin Infect Dis 41: 1297-1303.
2. Hoel S, Mehli L, Bruheim T, Vadstein O, Jakobsen AN (2015) Assessment of microbiological quality of retail fresh sushi from selected sources in Norway. J Food Protect 78: 977-982.
3. Tan Y, Haresh K, Chai L, Ghazali F, Son R (2008) Prevalence of Campylobacter spp. in retailed ready-to-eat sushi. Int Food Res J 15: 331-336.
4. Barralet J, Stafford R, Towner C, Smith P (2004) Outbreak of Salmonella Singapore associated with eating sushi. Commun Dis Intell Q Rep 28: 527.
5. Jain S, Chen L, Dechet A, Hertz AT, Brus DL, et al. (2008) An outbreak of enterotoxigenic Escherichia coli associated with sushi restaurants in Nevada, 2004. Clin Infect Dis 47: 1-7.
6. Thompson C, Wang Q, Bag S, Franklin N, Shadbolt C, et al. (2017) Epidemiology and whole genome sequencing of an ongoing point-source Salmonella Agona outbreak associated with sushi consumption in western Sydney, Australia 2015. Epidemiol Infect 145: 2062-2071.
7. Atanassova V, Reich F, Klein G (2008) Microbiological quality of sushi from sushi bars and retailers. J Food Protect 71: 860-864.
8. Diplock K (2003) The potential of sushi rice to serve as a medium for bacterial growth. Environ Health Rev 1: 109-116.
9. Millard G, Rockliff S (2003) Microbiological quality of sushi. ACT Department of Health. Retrieved February 11: 2008.
10. Puah, SM, Chua KH, Tan JAMA (2017) Prevalence of Staphylococcus aureus and Salmonella enterica in ready-to-eat sushi and sashimi. Trop Biomed 34: 45-51.
11. Kim, HJ, Lee HJ, Lee KH, Cho JC (2012) Simultaneous detection of Pathogenic Vibrio species using multiplex real-time PCR. Food Control 23: 491-498.
12. Novotny L, Dvorska L, Lorencova A, Beran V, Pavlik I (2004) Fish: a potential source of bacterial pathogens for human beings. Veterinarni Medicina, 49: 343-358.
13. Eschbach E, Martin A, Huhn J, Seidel C, Heuer R, et al. (2017) Detection of enteropathogenic Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus: performance of real-time PCR kits in an interlaboratory study. Eur Food Res Technol 243: 1335-1342.
14. Food and Drug Administration (2011) Fish and fishery products hazards and controls guidance. US Department of Health and Human Services Food and Drug Administration Center for Food Safety and Applied Nutrition.
15. Hastein T, Hjeltnes B, Lillehaug A, Utne Skare J, Berntssen M, et al. (2006) Food safety hazards that occur during the production stage: challenges for fish farming and the fishing industry. Rev Sci Tech 25: 607-625.
16. Su YC, Liu C (2007) Vibrio parahaemolyticus: a concern of seafood safety. Food Microbiol 24: 549-558.
17. Dickinson G, Lim KY, Jiang SC (2013) Quantitative microbial risk assessment of pathogenic *vibrios* in marine recreational waters of Southern California. Appl Environ Microbiol 79: 294-302.
18. Oliver J (2005) Wound infections caused by Vibrio vulnificus and other marine bacteria. Epidemiol Infect 133: 383-391.
19. Paydar M, Teh CSJ, Thong KL (2013) Prevalence and characterisation of potentially virulent Vibrio parahaemolyticus ináseafood in Malaysia using conventional methods, PCR and REP-PCR. Food Control 32: 13-18.
20. Authority NF (2007) Food safety guidelines for the preparation and display of sushi. NSW Food Authority.
21. Xu YG, Sun LM, Wang YS, Chen PP, Liu ZM, et al. (2017) Simultaneous detection of Vibrio cholerae, Vibrio alginolyticus, Vibrio parahaemolyticus and Vibrio vulnificus in seafood using Dual Priming Oligonucleotide (DPO) system-based multiplex PCR assay. Food Control 71: 64-70.
22. Köppel R, Tolido I, Marti G, Peier M (2017) Detection of DNA from Escherichia coli, Clostridium perfringens, Staphylococcus aureus and Bacillus cereus after simplified enrichment using a novel multiplex real-time PCR system. Eur Food Res Technol 243: 521-530.
23. Blanco-Abad V, Ansede-Bermejo J, Rodriguez-Castro A, Martinez-Urtaza J (2009) Evaluation of different procedures for the optimized detection of Vibrio parahaemolyticus in mussels and environmental samples. Int J Food Microbiol 129: 229-236.
24. Li R, Chiou J, Chan EWC, Chen S (2016) A novel PCR-based approach for accurate identification of Vibrio parahaemolyticus. Front Microbiol 7: 44.
25. Neogi S, Chowdhury N, Asakura M, Hinenoya A, Haldar S, et al. (2010) A highly sensitive and specific multiplex PCR assay for simultaneous detection of Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulnificus. Lett Appl Microbiol 51: 293-300.
26. Wei S, Zhao H, Xian Y, Hussain MA, Wu X (2014) Multiplex PCR assays for the detection of Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio vulnificus, and Vibrio cholerae with an internal amplification control. Diagn Micr Infec Dis 79: 115-118.
27. Lin CS, Lin TS, Yu DY, Su YC, Tsai YH (2016) Identification of Vibrio parahaemolyticus in Seafood by Multiplex PCR. J Aquat Food Prod T 25: 1301-1310.
28. Luan X, Chen J, Liu Y, Li Y, Jia J, et al. (2008) Rapid quantitative detection of Vibrio parahaemolyticus in seafood by MPN-PCR. Curr Microbiol 57: 218-221.
29. Machado A, Bordalo AA (2016) Detection and quantification of Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio vulnificus in coastal waters of Guinea-Bissau (West Africa). EcoHealth 13: 339-349.
30. Ubong A, Tunung R, Noorlis A, Elexson N, Tuan Zainazor T, et al. (2011) Prevalence and detection of Vibrio spp. and Vibrio cholerae in fruit juices and flavored drinks. Int Food Res J 18: 1163-1169.
31. Cantet F, Hervio-Heath D, Caro A, Le Mennec C, Monteil C, et al. (2013) Quantification of Vibrio parahaemolyticus, Vibrio vulnificus and Vibrio cholerae in French Mediterranean coastal lagoons. Res Microbiol 164: 867-874.
32. Fukushima H, Seki R (2004) Ecology of Vibrio vulnificus and Vibrio parahaemolyticus in brackish environments of the Sada River in Shimane Prefecture, Japan. Fems Microbiol Ecol 48: 221-229.
33. Bonny SQ, Hossain MAM, Lin TK, Ali ME (2018) Multiplex MPN-PCR for the enumeration of three major *Vibrios* in raw fishes in Malaysia. Food Control 90: 459-465.

34. Garrido-Maestu A, Vieites-Maneiro R, Peñaranda E, Cabado AG (2015) Development, and complete evaluation, of a novel Most-Probable-Number (MPN) qPCR method for accurate and express quantification of Listeria monocytogenes in foodstuffs. Eur Food Res Technol 241: 697-706.

35. Hossain MAM, Ali ME, Hamid SBA, Asing, Mustafa S, et al. (2017) Targeting double genes in multiplex PCR for discriminating bovine, buffalo and porcine materials in food chain. Food Control 73: 175-184.

36. Teh C, Chua KH, Puthucheary SD, Thong KL (2008) Further evaluation of a multiplex PCR for differentiation of Salmonella paratyphi A from other salmonellae. Jpn J Infect Dis 61: 313-314.

37. Thong KL, Teh C, Chua K (2014) Development and evaluation of a Multiplex Polymerase Chain Reaction for the detection of Salmonella species. Trop Biomed 31: 689-697.

38. Hara-Kudo Y, Nishina T, Nakagawa H, Konuma H, Hasegawa J, et al. (2001) Improved method for detection of Vibrio parahaemolyticus in seafood. Appl Environ Microbiol 67: 5819-5823.

39. Blodgett R (2010) BAM appendix 2: most probable number from serial dilutions. Bacteriological analytical manual. Food and Drug Administration, Silver Spring, MD.

40. Lim BK, Thong KL (2009) Application of PCR-based serogrouping of selected Salmonella serotypes in Malaysia. J Infect Dev Countr 3: 420-428.

41. Cañigral I, Moreno Y, Alonso JL, González A, Ferrús MA (2010) Detection of Vibrio vulnificus in seafood, seawater and wastewater samples from a Mediterranean coastal area. Microbiol Res 165: 657-664.

42. Teh C, Chua K, Thong KL (2010) Simultaneous differential detection of human pathogenic and nonpathogenic Vibrio species using a multiplex PCR based on gyrB and pntA genes. J Appl Microbiol 108: 1940-1945.

43. Chang CH, Lin HY, Ren Q, Lin YS, Shao KT (2016) DNA barcode identification of fish products in Taiwan: Government-commissioned authentication cases. Food Control 66: 38-43.

44. Lorentzen G, Breiland MSW, Cooper M, Herland H (2012) Viability of Listeria monocytogenes in an experimental model of nigiri sushi of halibut (Hippoglossus hippoglossus) and salmon (Salmo salar). Food Control 25: 245-248.

45. Zijlstra C, Van Hoof R (2006) A multiplex real-time polymerase chain reaction (TaqMan) assay for the simultaneous detection of Meloidogyne chitwoodi and M. fallax. Phytopathology 96: 1255-1262.

46. Manitoba (2013) Food Safety Guidelines for the Preparation of Sushi, Manitoba Health.

47. Senderovich Y, Izhaki I, Halpern M (2010) Fish as reservoirs and vectors of Vibrio cholerae. PLoS One 5: e8607.

48. Angelidis A, Chronis E, Papageorgiou D, Kazakis I, Arsenoglou K, et al. (2006) Non-lactic acid, contaminating microbial flora in ready-to-eat foods: A potential food-quality index. Food Microbiol 23: 95-100.

49. Børresen T (2008) Improving seafood products for the consumer: Elsevier.

50. Randa MA, Polz MF, Lim E (2004) Effects of temperature and salinity on Vibrio vulnificus population dynamics as assessed by quantitative PCR. Appl Environ Microbiol 70: 5469-5476.

51. Hara-Kud Y, Miwa N, Yamasaki S, Yatsuyanagi J, Iwade Y, et al. (2005) Quantitative detection of Vibrio vulnificus in seafood. Kansenshogaku zasshi. The Journal of the Japanese Association for Infectious Diseases 79: 931-936.

52. Liang WL, Pan YL, Cheng HL, Li TC, Yu PHF, et al. (2016) The microbiological quality of take-away raw salmon finger sushi sold in Hong Kong. Food Control 69: 45-50.

53. Suppin D, Rippel-Rachle B, Schopf E, JM F (2002) A survey of the microbiological condition of sushi from Viennese retail operations. Food Safety Assurance and Veterinary Public Health: Safety assurance during food processing 377.

54. Hassan R, Tecle S, Adcock B, Kellis M, Weiss J, et al. (2018) Multistate outbreak of Salmonella Paratyphi B variant L (+) tartrate (+) and Salmonella Weltevreden infections linked to imported frozen raw tuna: USA, March–July 2015. Epidemiol Infect 146: 1461-1467.