Prevalence and detection of *Vibrio* spp. and *Vibrio cholerae* in fruit juices and flavored drinks

[1,*]Ubong, A., [1]Tunung, R., [1]Noorlis, A., [1]Elexson, N., [2]Tuan Zainazor, T. C.,
[1]Ghazali, F. M., [3]Nakaguchi, Y., [3]Nishibuchi, M. and [1]Son, R.

[1] *Centre of Excellence for Food Safety Research, Faculty of Food Science and Technology,*
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor Darul Ehsan, Malaysia
[2]*National Public Health Laboratory, Malaysian Ministry of Health, Lot 1835 Kampung*
Melayu, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia
[3]*Center for Southeast Asian Studies, Kyoto University, Kyoto 606-8501, Japan*

Abstract: In this study, the prevalence of *Vibrio* spp. and *V. cholerae* in flavored drinks and fruit juices sold at hawker stalls and in restaurants were determined using the most probable number (MPN) method and polymerase chain reaction (PCR) assay. One hundred and twenty drinks samples of four types of drinks (Iced Milk Rose Syrup, n=25; Iced Milk Corn Syrup, n=20; Apple Juice, n=37 and Iced Carrot Milk, n=28) were collected at two different settings; mainly hawker stalls and restaurants. Upon analysis, the prevalence of *Vibrio* spp. and *V. cholerae* in fruit juices and flavored drinks for hawker stalls were 93.3% and 36.7%, respectively whereas for the restaurants, were 93.3% and 20% respectively. Overall, the prevalence of *Vibrio* spp. and *V. cholerae* in all the drink samples were 93.3% and 28.3% respectively, highlighting the needs for the improvement of the hygiene and sanitation practice in the settings studied.

Introduction

V. cholerae, the causative agent of cholera disease has been a major health concern worldwide. Cholera outbreak had started as early as during the ancient civilization which was located in Ganges delta, India (Barua, 1992; Prouty and Klose, 2006). Pathogencity of *V. cholerae* is due to their cholera toxin gene (*ctx*) (Finkelstein *et al*., 1963; Broeck *et al*., 2007). Despite of having this virulence gene, even so, only a small number of *V. cholera* is capable of producing cholera toxin and give rise to cholera clinical symptom (Nishibuchi, 2006). To date, cholera disease affects most third world populations in which the manifestation of the disease is characterized by acute diarrhea, often described in other name as 'rice water' stool. Cholera epidemics were mainly associated with *V. cholerae* O1 serotype but the non-O1 strains are less significant to the epidemics (Radu *et al*., 2002). Nevertheless, from previous reports, foods were also found to be contaminated with *V. cholerae*. Other sources of infection include seafood, fruits and vegetables. The alarming effect of cholera has raised concern to the public as cholera incidences

not only affects public well being, but in a way, also has significant impact to the economic sector (Robin, 2007).

Polymerase Chain Reaction (PCR) had been used to a great extends in researches to amplify targeted DNA or gene. Meanwhile, Most Probable Number (MPN) test applies the use of statistical mathematics to obtain quantitative data on concentration of bacteria in a sample. For that reason, MPN test and PCR techniques were combined together to detect the presence of bacteria of interest and following that, to estimate the bacterial count in a sample. In fact, coupled MPN-PCR was proven to be successful in researches in food laboratory analysis, practically involving quantification and enumeration of microorganism in food samples (Mäntynen, 1996; Hara-Kudo *et al*., 2003; Gomez-Gil and Roque, 2006).

To study more on prevalence of *V. cholerae* in beverages, this research focused on the occurrence of *V. cholerae* associated with fruit juices and flavored drinks from hawker stalls and restaurants in Serdang, Selangor. Hence, collected data will assist towards comprehending the risk of acquiring *V. cholerae* from

these drinks.

Materials and Methods

Sample type

The drinks samples analyzed in this research includes 35 samples of iced milk rose syrup (*air bandung ais*), 20 samples of milk corn syrup (*air jagung ais*), 37 samples of iced apple juice (*jus epal ais*) and 28 samples of iced carrot milk (*jus lobak merah ais*).

Sampling

One hundred and twenty drink samples were purchased from hawker stalls and restaurants at four different locations, mainly in Serdang, Selangor, Malaysia (Table 1). In general, the hawker stalls were located by the roadside with improper water supplies. Meanwhile, the restaurants were located between rows of shop houses with proper water supplies. Methods applied in sampling were done according to the standard method of Bacteriological Analytical Manual (2004). Modifications of the standard method were done according to Hara-Kudo *et al*. (2001), Chai *et al*. (2008) and Tunung *et al*. (2010), as well as referring to methods used by Radu *et al*. (2002).

Most Probable Number test

Two 50 ml centrifuge tubes were filled with 50 ml drink sample each and centrifuged at 11,200 x g for 10 min. Following that, 45 ml supernatant from each tube were discarded. The pellets were resuspended with a vortex mixer. Next, 5 ml aliquot from each centrifuge tubes were mixed with 90 ml TSB with 1% NaCl and stomached for two min using a stomacher (Interscience, France) to homogenize the solution. Salt Polymyxin Broth (SPB; Nissui, Japan) was used for dilutions of 100-fold and 1000-fold of the stomacher fluid prior to MPN three tubes test. 1 ml of each dilution was pipette into three tubes and incubated at 37°C for 18 to 24 hours.

Preparation of genomic DNA

Genomic DNA from the MPN tubes was extracted using boil cell method, as described by Tunung *et al*. (2010) and Kawasaki *et al*. (2005). MPN tubes that turned turbid after incubation were centrifuged at 13,400 x g for 1 min, the supernatants were discarded and 500 μl of distilled water was added to the tubes to resuspend the pellet. After that, boiling and immediate cooling of the tubes were done each at 10 minutes, respectively. Finally, the tubes were centrifuged again at 13,400 x g for 3 min. The clear supernatants were transferred to sterile new microcentrifuge tubes to be kept at -20°C.

Genomic DNA amplification by PCR

Specific forward (Vspp-16SF-156-5'-CGTAAAGCGCATGCAGGTG-3') and reverse (Vspp-16SR-157-5'-CTTCGCCACCGGTATTCCTT-3') primer pairs (Gonzalez-Escalona *et al*., 2005) and forward (VCT1 -5'-ACAGAGTGAGTACTTTGACC-3') and reverse (VCT2-5'-ATACCATCCATATATTTGGGAG-3') primer pair (Hoshino *et al*., 1998) were used to detect *Vibrio* spp. and *V. cholerae*. Amplification of genomic DNA with two sets of forward and reverse primers was performed on a thermocycler (Applied Biosystems 2720 Thermal Cycler, USA). Two μl of DNA boil lysate from MPN tubes were added to PCR mixture which made up a 20 μl reaction mixture (Tunung *et al*., 2009); 4.0 μl of 5× PCR Buffer, 0.2 mM of $MgCl_2$, 0.25 mM of dNTPs mix, 0.25 μM of *Vibrio* spp. and *V. cholerae* forward and reverse primers, and 1 U/μl of *Taq* polymerase. Amplification condition used was 4 min at 96°C for pre-denaturation; following that, a 35 cycles of denaturation at 94°C for 45 s, annealing at 59°C for 45 s, extension at 72°C for 45 s and a final round of extension at 72°C for 7 min. The PCR products were electrophoresed on 1% (w/v) agarose gel in 5× TBE Buffer for 23 min at 100 V and visualized under ultraviolet light using computer software (Gel Documentation System, SynGene, UK).

Table 1. Occurrence of *Vibrio* spp. and *V. cholerae* in drink samples

Drinks	Hawker Stalls		Restaurants		TOTAL	
	[a]Vs[+]/n, (%)[e]	[b]Vc[+]/n, (%)[e]	[a]Vs[+]/n[a], (%)[e]	[b]Vc[+]/n, (%)[e]	[c]Vs[+]/n, (%)[e]	[d]Vc[+]/n, (%)[e]
Iced Milk Rose Syrup	15/15 (100)	5/15 (33)	20/20 (100)	5/20 (25)	35/35 (100)	10/35 (28.6)
Iced Corn Syrup	20/20 (100)	8/20 (40)	_[f]	_[f]	20/20 (100)	8/20 (40)
Iced Apple Juice	11/15 (73.3)	8/15 (53.3)	18/22 (81.8)	2/22 (20)	29/37 (78.4)	10/37 (27)
Iced Carrot Milk	10/10 (100)	1/10 (10)	18/18 (100)	5/18 (40)	28/28 (100)	6/28 (21.4)
TOTAL	**56/60 (93.3)**	**22/60 (36.7)**	**56/60 (93.3)**	**12/60 (20)**	**112/120 (93.3)**	**34/120 (28.3)**

[a] Positive PCR for *Vibrio* spp./number of drink samples
[b] Positive PCR for *V. cholerae*/number of drink samples
[c] Total positive PCR for *Vibrio* spp./total number of drink samples
[d] Total positive PCR for *V. cholerae* /total number drink samples
[e] Percentage (%)
[f] Sample not available

Isolation and confirmation pure cultures

MPN tubes which showed turbid suspension were streaked on CHROMagar™ Vibrio (Paris, France). Suspected blue colonies of *V. cholerae* were picked after 18-24 hours of incubation and cultured in Tryptic Soy Agar (TSA; Merck, Germany) slant with 1% sodium chloride (NaCl; Merck, Germany). The suspected *V. cholerae* isolates were confirmed again through PCR using specific *V. cholerae* primers. Forward and reverse primers used were *ompW* (Nandi *et al.*, 2000).

Screening of V. cholerae *O1 and O139 using PCR*

Confirmed *V. cholerae* isolates were subjected to *V. cholerae* O1 and O139 screening which were carried out with *V. cholerae* O1 specific forward and reverse primers, O1F2-1 [5'-GTTTCACTGAACAGATGGG-3'] and O1R2-2 [5'-GGTCATCTGTAAGTACAAC-3'] respectively; *V. cholerae* O139 specific forward and reverse primers, O139F2 [5'-AGCCTCTTTATTACGGGTGG-3'] and O139R2 [5'-GTCAAACCCGATCGTAAAGG-3']. Amplification of genomic DNA was performed on a thermocycler (Applied Biosystems 2720 Thermal Cycler, USA). A 2 μl DNA boil lysate from samples were added to PCR mixture which made up into 20 μl reaction mixture (Tunung *et al.*, 2010); 4.0 μl of 5× PCR Buffer, 1.5 mM of $MgCl_2$, 0.21 mM of dNTPs mix, 0.5 μM of *V. cholerae* O1 forward and reverse primers, 0.27 μM of *V. cholerae* O139 forward and reverse primers and 0.75 U/μl of *Taq* polymerase. Amplification condition used was 5 min at 94°C for pre-denaturation; following that, a 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and finally, a final extension at 72°C for 7 min. The PCR products were electrophoresed on 1% (w/v) agarose gel in 5× TBE Buffer for 23 min at 100 V and visualized under ultraviolet light using computer software (Gel Documentation System, SynGene, UK).

Results

PCR assay enables the detection of *Vibrio* spp. and *V. cholerae* in drink sample using their specific primers. This DNA amplification method produced PCR products of 162 bp and 308 bp for *Vibrio* spp. and *V. cholerae* respectively. Prevalence of *Vibrio* spp. and *V. cholerae* in drink samples from hawker stalls and restaurants is shown in Table 1. The prevalence of *Vibrio* spp. in the drink samples from hawker stalls was 93.3%. *Vibrio* spp. was most predominant in the iced milk rose syrup, iced milk corn syrup and iced milk carrot milk, each at 100% prevalence. *Vibrio*

spp. was least predominant in apple juice (73.3%). In samples obtained from the restaurants, the prevalence of *Vibrio* spp. was 93.3%, showing highest detection in iced milk rose syrup, iced apple juice and iced carrot milk, each at 100% prevalence percentage.

The prevalence of *V. cholerae* in the drink samples from hawker stalls was 36.7 %. *V. cholerae* was most predominant in iced milk corn syrup (40%), but was least predominant in iced carrot milk (10%). In samples obtained from the restaurants, the prevalence of *V. cholerae* was 20%, showing highest detection in iced milk rose syrup and iced carrot milk, each at 40% prevalence level.
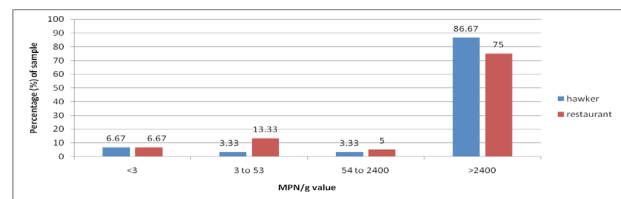

Figure 1. Percentage distribution of MPN value of *Vibrio* spp. in fruit juices and flavored drinks
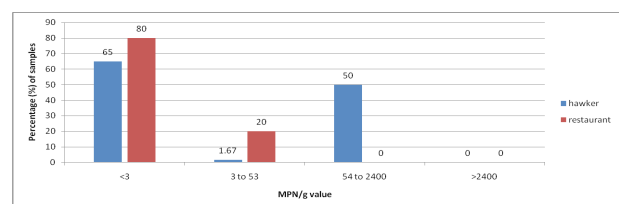

Figure 2. Percentage distribution of MPN value of *V. cholerae* in fruit juices and flavored drinks

Table 2 summarizes the density of *Vibrio* spp. and *V. cholerae* in the drink samples. The analysis of 120 drink samples showed that the density of *Vibrio* spp. ranged from <3 MPN/g to >2400 MPN/g, indicating a broad density range. However, majority of the drinks samples had MPN value distribution of >2400 MPN/g. Data analysis showed that not more than 6.67% drink samples from both hawker stalls and restaurants contained <3 MPN/g *Vibrio* spp. Meanwhile, 13.33% and below of the samples from both locations contained 3 to 53 MPN/g of *Vibrio* spp. MPN/g value of 54 to 2400 only showed 3.33% *Vibrio* spp. in samples from hawker stalls while 5% *Vibrio* spp. in samples from restaurants. More than 70% of the samples from both locations contained >2400 MPN/g *Vibrio* spp.

MPN distribution for *V. cholerae* ranged from <3 MPN/g to 2400 MPN/g with majority of the drink samples had distribution of <3 MPN/g. Up to sixty percent and 80% of the drink samples from both hawker stalls and restaurants contained <3 MPN/g *V. cholerae*. For MPN value of 3 MPN/g to 53

Table 2. Densities (MPN/g) of *Vibrio* spp. and *V. cholerae* in drink samples

Drinks	Hawker Stalls						Restaurants					
	Vibrio spp.			*V. cholerae*			*Vibrio* spp.			*V. cholerae*		
	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max
Milk Rose Syrup	>2400	>2400	>2400	<3	<3	9.4	6	>2400	>2400	<3	<3	11
Milk Corn Syrup	>2400	>2400	>2400	<3	<3	9.3	[d]-	[d]-	[d]-	[d]-	[d]-	[d]-
Apple Juice	<3	1100	>2400	<3	3	93	<3	>2400	>2400	<3	<3	16
Carrot Milk	>2400	>2400	>2400	<3	<3	3	460	>2400	>2400	<3	<3	9.1
Min/Med/Max	<3	>2400	>2400	<3	<3	93	<3	>2400	>2400	<3	<3	16

[a]Min   = Minimum MPN/g value
[b]Med   = Median MPN/g value
[c]Max   = Maximun MPN/g value
[d]-   = Sample not available

MPN/g showed 1.67% and 20% of *V. cholerae* in the drink samples from hawker stalls and restaurants, respectively. Only drink samples from hawker stalls contained 54 MPN/g to 2400 MPN/g *V. cholerae*.

Cholera toxin gene primers were used in this research to sift through pathogenic *V. cholera* in which these *V. cholerae* carry *ctxA* gene, a type of cholera toxin responsible for cholera outbreak. Hence, the results analysis showed that the total prevalence of *ctxA* carriers in the drink samples was 28.3% with density range of <3 MPN/g to >2400 MPN/g (Table 1 and Table 2). Thirty-eight *Vibrio* spp. isolates were obtained using the MPN-Plating technique and the isolates were screened for the presence of *V. cholerae* O1 and O139 serogroups. However, none of the O1 and O139 serogroups were detected in any of the drink samples.

Discussions

MPN techniques promote the growth of desired bacteria of study prior to the sensitive detection of PCR method. Basically, this is achieved by using the enrichment broth (TSB with 1% NaCl) in the MPN three tubes to promote the growth of *V. cholerae*. The 1% w/v salt content of TSB supports the growth of *Vibrio* spp. and *V. cholerae* due to the capability of most vibrios growing at high or sometimes low concentrations of NaCl (Gomez-Gil and Roque, 2006). The coupled MPN-PCR method has been used in various studies for the rapid and sensitive detection of foodborne pathogens in the local setting in Selangor, Malaysia (Chai *et al*., 2008; Tunung *et al*., 2009; Lee *et al*., 2009; Jeyaletchumi *et al*., 2010; Suzita *et al*., 2010; Usha *et al*., 2010; Wong *et al*., 2011; Noorlis *et al*., 2011).

As described in Table 1, prevalence of *Vibrio* spp. from both locations has the same MPN value which was 93.3%. Meanwhile, the prevalence of *V. cholerae* in drinks from hawker stalls and restaurants were 36.7% and 20% respectively. *Vibrio* spp. and *V. cholerae* are widely spread in freshwater and estuarine regions (Nishibuchi, 2006). However, preparation of

the drink samples involved many stages and it is not impossible that certain stages were contaminated with *Vibrio* spp. and *V. cholerae* due to poor food handling, storage, as well as lacking in hygienic practice.

V. cholerae requires a warmer temperature to grow and reproduce as it is one of the disease-causing pathogens in human, which grow at body temperature (Urakawa and Rivera, 2006). During sampling process, we also carried out some observation at sampling locations. Prior to sale, drinks at hawker stalls were prepared before display. They were often left unattended and exposed under sunlight for quite a while before they were purchased by the customers. After much time, the bottom layer of the container containing the drinks slowly becoming warmer and the upper layer was still immersed in ice, possibly providing the microbes an environment to multiply (Berger *et al*., 1992; LeChevallier *et al*., 1991, 1993).

Some hawker stalls do not cover the plastic containers, allowing dust particles and even airborne microbes to settle down on the surface of the drinks; often hawker stalls were set up at places where there were many passersby and at the roadside. Apart from that, hawker stalls do not have proper kitchen and water supplies compared to restaurants. The chopping board that were used for slicing were rinsed only once in a while in water collected in a washbasin.

In the same fashion, drinks samples from restaurants also harbored *V. cholerae*. Cross-contamination could possibly be acquired from kitchen utensils and food handlers (Chai *et al*., 2008; Tunung *et al*., 2009). Through much observations, food handlers at the restaurants paid less attention to personal hygiene; not wearing the proper attire whilst preparing drinks. Previous study done by Ackers *et al*. (1997) described that *V. cholera* O1 was found in cantaloupe melon which was contaminated during slicing and handling by asymptomatic carrier. Likewise, fruits could be contaminated during harvesting or stumble upon direct contact with contaminated soil.

Nevertheless, by taking the content of the drinks

into account, this factor might also influence the presence of *Vibrio* spp. and *V. cholerae* in a particular drink. *Vibrio* spp. and *V. cholerae* were most prevalent in iced milk rose syrup, iced corn syrup and iced carrot milk (Table 2) compared to iced apple juice. Considering the fact that milk was alkaline, *Vibrio* spp. and *V. cholerae* favors these drinks as they might provide suitable condition for their growth. In fact, Gomez-Gil and Roque (2006) described that most vibrios have the ability to grow at pH values above 8.0. In spite of this, *V. cholerae* also showed slightly higher prevalence in iced apple juice compared to the other drinks. Being the main component of apple, glucose might as well provide *V. cholerae* with substrate for its metabolism which promotes their growth apple juices. In fact, it was described in previous findings that vibrios also perform glucose fermentation (Desmarchelier, 2003).

According to Ackers *et al.* (1997), vibrios contaminating the rind of the fruits will not survive for more than a few days. However, according to Mcdougald and Kjelleberg (2006), vibrios are capable of developing adaptive response to low nutrient condition. Marine vibrios that were found to be starved cells were able to change shape from rods to cocci and maintained the normal cell structures, as well as continue to remain viable for the next 2.5 years. Vibrios changes from their normal size into ultramicrocells in respond to starvation conditions. Starvations in Vibrios are normally characterized by reduction in cell volume, DNA and ribosome content and protein synthesis rate. Hence, a smaller cell size enhance nutrient uptake due to the increase in surface-to-volume ratio and also allowing the cell to escape predation.

In general, cholera toxin gene confers the virulence factor of *V. cholerae*, giving this bacterium the ability to cause disease in human. This gene was normally found in *V. cholerae* of the O1 and 0139 serogroups. The purpose of detecting *ctxA* during MPN-PCR was to analyze whether *V. cholerae* from the drink samples might possess cholera toxin gene. The detection of *V. cholerae* had brought up the concern that these drinks might have the possible risk to public well being upon consumption. Hence, it is important to supervise the practice of hygiene as microorganisms should be treated as potentially pathogenic at any level. In any case, a more detailed research is required for a better understanding on *V. cholerae* and its prevalence in fruit juices and flavored drinks.

Acknowledgement

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