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Enumeration of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in fruit juices

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Abstract: *Salmonella* has caused foodborne illnesses globally and it has been a rising threat on fresh produce. The objective of this study was to determine the prevalence and concentration of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in freshly prepared fruit juice sold at hawker stalls. Analysis was conducted by employing most probable number-polymerase chain reaction (MPN-PCR). A total of 50 freshly prepared fruit juices were examined and the prevalence of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in the fruit juices were 34%, 20% and 10%, respectively, with an estimated microbial load varying from 0 to 42 MPN/g. Of the five different fruits, carrot juice had the highest prevalence of *Salmonella* spp. (60%) and *Salmonella* Typhi (40%). However, *Salmonella* Typhimurium was detected in apple (30%), orange (10%) and starfruit juice (10%). Factors contributing to the presence of *Salmonella* were cross-contamination and poor sanitation practice. Besides, negligence on temperature and storage time also led to the growth of *Salmonella*. Proper monitoring and risk assessment are needed in order to establish control measures to ensure the quality and safety of fruit juices in Malaysia.

Keywords: *Salmonella* Typhi, *Salmonella* Typhimurium, MPN-PCR, fresh fruit juice

Introduction

In many countries, the consumption of ready-to-eat fresh fruits is on the rise due to its rich vitamins, minerals and fibres. Therefore, attention has been focused on foodborne diseases associated with fresh fruits and vegetables (IFPA, 2000). Due to the higher demand of these ready-to-eat fresh fruits, there has been an increase in the production of minimally processed food (IFPA, 2000; Lin *et al*., 2002). The Centres for Disease Control and Prevention (CDC, 2009) revealed that an estimated of 12.3% of total foodborne diseases outbreaks from 1990 to 2007 were related to fruits and vegetables. This might be attributed to the cross-contamination of fresh fruits and vegetables from soil, insects, animals and humans which may occur during agricultural practices such as processing, distribution and storage (Ukuku *et al*., 2001; Beuchat, 2002). In Malaysia, we have reported the presence of foodborne pathogens in various types of raw food samples (Rusul *et al*., 1996; Son *et al*., 1998; Son *et al*., 1998; Noorzaleha *et al*., 2003; Chai *et al*., 2007; Jeyaletchumi *et al*., 2010; Tunung *et al*., 2011; Wong *et al*., 2011).

Parish *et al*. (1997) demonstrated that in the past, fruit juices had been excluded from food safety issues due to their inherent low pH. However, Salmonella are chemoorganotrophic and non-fastidious microorganisms which inherit the ability to adapt to wide pH of approximately 4.0 to 9.0 and temperature ranges of 5°C to 47°C, respectively (Jay, 2000). Hence, without proper refrigeration or pasteurization, the fresh fruits are at maximum risk of causing salmonellosis. Given the hazardous impact of *Salmonella* on human, the present was designed to determine the prevalence and concentration of *Salmonella* spp., *Salmonella* Typhimurium and *Salmonella* Typhi in fruit juices.

Materials and Methods

Sample preparation

From August to November 2010, 50 fresh fruit juice samples (Table 1) prepared from mechanical squeezing were collected randomly from 10 fruit juice vendors and hawker stalls in Serdang and the Klang Valley, Malaysia. The samples were placed in an ice box immediately after collection. Within 2 h, the samples were examined.

Table 1. Fruit types and the number of fruit juices examined for the prevalence and concentration of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium.

English name	Local name	Scientific name	Number of samples (n)
Orange	Oren	*Citrus sinensis*	10
Apple	Epal	*Malus domestica*	10
Watermelon	Tembikai	*Citrullus lanatus*	10
Starfruit/ Carambola	Belimbing	*Averrhoa carambola*	10
Carrot	Carot	*Daucus carota*	10
Total			50

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Pre-enrichment and MPN method

A 10 ml of samples were aseptically transferred into 90 ml of buffered peptone water (BPW; Merck, Darmstadt, Germany) in a stomacher bag and incubated for 6 hours at 37°C. These pre-enriched samples were introduced to a three-tube most probable number (MPN) method. The serial dilutions of fluid into 100 and 1000 fold were performed. All the tubes were incubated at 37°C for 24 h. After 24 h, those turbid tubes were subjected to further analysis using multiplex PCR for the detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium.

DNA Extraction

DNA was extracted from turbid MPN tubes by the boiled cell method (Pui *et al*., 2011). A 1 ml of each broth was centrifuged at 12,000 rpm for 3 min. The supernatant was then discarded whereas the pellet was suspended in 500 µl of sterile distilled water and vortexed. The cell suspension was boiled for 10 min and immediately cooled at -20°C for 10 min, followed by centrifugation for 3min at 12,000 rpm. The supernatant was transferred as DNA template solution for subsequent multiplex PCR analysis.

Multiplex PCR

Three sets of primers which consist of ST11 and ST15 specific to *Salmonella* spp., sty-1 and sty-2 specific to *Salmonella* Typhi as well as Fli15 and Typ04 specific to *Salmonella* Typhimurium (Table 2) were used. All oligonucleotide primers were synthesized by 1st BASE Laboratories, Malaysia. Positive controls in the multiplex PCR assay used *Salmonella* Typhi and *Salmonella* Typhimurium strains from Institute for Medical Research, Malaysia. Multiplex PCR was conducted using Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The optimized multiplex PCR was performed in a 50 µl master mix containing 10 µl of 5x PCR buffer, 1 µL of dNTP mix, 1 µl each of ST11 and ST15, 6 µl each of Fli15, Typ04, sty-1 and sty-2, 5 µL of $MgCl_2$, 3.7 µl of sterile distilled water, 4 µl of DNA template and 0.3 µl of *Taq* DNA Polymerase. All ingredients used in the master mix were purchased from Vivantis Technologies, Selangor, Malaysia. The thermocycler conditions were initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. An amount of 5 µl of the PCR product was loaded on a 1.2% (w/v) agarose gel using 0.5x TBE buffer with 1 µl of ethidium bromide. The gel was run at 90 V for 40 min before visualized under ultraviolet light.

Results and Discussion

A total of 50 fresh fruit juices were analyzed for the prevalence of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium using the amplicons at 429 bp, 300 bp and 620 bp respectively. The representative gel electrophoresis image of the various combination of PCR primer sets amplicons of fresh fruit juice extracts were shown in Figure 1. In the present study, the prevalence of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in total fruits were 34%, 20% and 10% respectively. The prevalence and microbial load data of five different fruits (orange, apple, watermelon, starfruit and carrot) from ten different hawker stalls located around Serdang and the Klang Valley are tabulated in Table 3 and 4. *Salmonella* spp. and *Salmonella* Typhi were detected in all five different fruits, whereas *Salmonella* Typhimurium was not detected in watermelon juice and carrot juice.

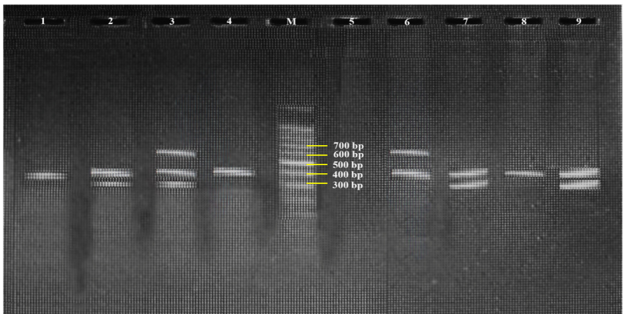

Figure 1. Gel electrophoresis image for the identification of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium. Lane 1 and 4 shows the PCR amplicons specific for *Salmonella* spp. at 429 bp. Lane 2 and 7 shows PCR amplicons specific to *Salmonella* spp. and *Salmonella* Typhimurium at 429 bp and 300 bp. Lane 3 shows PCR amplicons specific for *Salmonella* spp., *Salmonella* Typhi, *Salmonella* Typhimurium at 429 bp, 300 bp and 620 bp respectively. Lastly, lane 6 shows the PCR amplicons specific to *Salmonella* spp. and *Salmonella* Typhi at 429 bp and 620 bp. Lane M shows the 100 bp DNA ladder, (9) positive control and (10) negative control.

Of the five different fruits, carrot juice had the highest prevalence of *Salmonella* spp. (60%) and *Salmonella* Typhi (40%). The prevalence of *Salmonella* Typhi in carrot juice was two times higher than orange juice and four times more prevalent than apple and watermelon juice, respectively. On the other hand, *Salmonella* Typhimurium was detected highest in apple juice (30%) followed by orange juice (10%) and starfruit juice (10%). Referring to Table 4, the estimated microbial load of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in fruit juices varied from 0 to 42.00 MPN/g. The concentration of *Salmonella* spp. and *Salmonella*

Table 2. Primer sequences used simultaneously in multiplex PCR.

Target species	Primer sets	Sequence 5' to 3'	Amplicon size (bp)	Reference
Salmonella spp.	ST 11	GCC AAC CAT TGC TAA ATT GGC CGA	429	Soumet *et al*., 1999
	ST15	GGT AGA AAT TCC CAG CGG GTA CTG G		
Salmonella Typhi	sty-1	TGC CGG AAA CGA ATC T	300	Zhu *et al*., 1996
	sty-2	GGT TGT CAT GCC AAT GCA CT		
Salmonella Typhimurium	Fli15	CGG TGT TGC CCA GGT TGG TAA T	620	Oliveira *et al*., 2002
	Typ04	ACT GGT AAA GAT GGC T		

Table 3. Prevalence of *Salmonella* spp*., Salmonella* Typhi and *Salmonella* Typhimurium in fruit juices from hawker stalls.

Fruit juices	*Salmonella* spp.		*Salmonella* Typhi		*Salmonella* Typhimurium	
	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
Orange juice	2/10	20.0	2/10	20.0	1/10	10.0
Apple juice	3/10	30.0	1/10	10.0	3/10	30.0
Watermelon juice	3/10	30.0	1/10	10.0	0/10	0.0
Starfruit/ Carambola juice	3/10	30.0	2/10	20.0	1/10	10.0
Carrot juice	6/10	60.0	4/10	40.0	0/10	0.0
TOTAL	17/50	34.0	10/50	20.0	5/50	10.0

[a]Number of positive samples/number of samples examined.
[b]Frequency (in %) of positive samples among the samples examined.

Table 4. Concentration of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium (MPN/g) in fruit juices from hawker stalls.

Fruit Juices	*Salmonella* spp.			*Salmonella* Typhi			*Salmonella* Typhimurium		
	Min[a]	Med[b]	Max[c]	Min[a]	Med[b]	Max[c]	Min[a]	Med[b]	Max[c]
Orange	0	0	9.20	0	0	6.0	0	0	6.10
Apple	0	0	9.20	0	0	6.20	0	0	9.20
Watermelon	0	0	12.00	0	0	6.20	0	0	0
Starfruit	0	0	9.40	0	0	6.20	0	0	3.00
Carrot	0	3.05	42.00	0	0	20.00	0	0	0

[a] Minimum MPN/g value
[b] Median MPN/g value
[c] Maximum MPN/g value

Typhi was highest in carrot juice with an estimated of 42.00 MPN/g and 20.00 MPN/g, respectively. The estimated concentration of *Salmonella* Typhimirium was relatively high in apple juice at 9.20 MPN/g. However, in all fruit juices, the minimum quantity and median quantity of *Salmonella* spp*., Salmonella* Typhi and *Salmonella* Typhimurium were 0 MPN/g, only *Salmonella* spp. in carrot juice had a median of 3.05 MPN/g.

In all five fruit juices examined, the results highlighted that *Salmonella* spp. (34%), *Salmonella* Typhi (20%) and *Salmonella* Typhimurium (10%) should be of concerned as the estimated microbial load has reached 42.00 MPN/g. As compared to many studies on *Salmonella* spp. detection in meat products, the isolation of *Salmonella* spp. in fruit juices in this study is of low levels (Gunaydin *et al*., 2006; Techathuvanan *et al*., 2010; Wang *et al*., 2010). The common pattern of consumption of meat is mostly after cooking with high temperature where vegetative cells are destroyed. However, in Malaysia, fruits are usually eaten raw or blended with other ingredients without heat treatment. So, it is noticeable that contracting salmonellosis becomes more vibrant when consuming contaminated fruits at its raw stage.

MPN-multiplex PCR method was employed in this study as it not only enables the enumeration to be completed in two days, but it can also equally detect and quantify *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in fruit juices. Even though PCR was incepted over 20 years ago, there is still a lack of data regarding its application and detection of foodborne pathogens in fresh fruits (Shearer *et al*., 2001). Studies conducted by Bhagwat (2004) on the detection of *Salmonella* in vegetable shown that substances in plant tissue or dead cells may inhibit PCR reaction. However, by incorporating the MPN method, the enrichment broth used helps to keep the substances of plants to a minimum and enhance the detection during PCR (Liming *et al*., 2004). Enrichment step in MPN method minimizes false positive results. This finding is supported by Pui *et al*. (2011) on sliced fruits, where it was reported that MPN-PCR complements multiplex PCR method and increases the accuracy of *Salmonella* detection.

The minimally processed fruits and vegetables are among the most possible source of foodborne pathogens (Han *et al*., 2000). The current trend of food consumption globally has seen increased mainly for

convenient, fresh and healthy beverages such as the fruit juices. It was proven that vegetables and fruits are reservoirs for foodborne salmonellae (Leverentz *et al*., 2001). With the rising threat of bacterial contamination on fresh produce, it becomes more important to enumerate the growth of *Salmonella* spp. and its serotypes in fruit juice. To our knowledge, no published study exists that describe the presence of *Salmonella* spp*., Salmonella* Typhi and *Salmonella* Typhimurium in fresh fruit juice in Malaysia.

These contamination of fresh produce may have been resulted from cross contamination or mishandling of fresh produce during harvesting, processing, transporting or during preparation before consumption. The farm to fork concept involves many parties during pre- and post-harvesting. A study conducted by Nutt *et al*. (2003) indicated that *Salmonella* Typhimurium isolated from poultry has the ability to survive and grow in a variety of fresh produce during pre-harvesting. The factors that influence *Salmonella*'s ability to cross contaminate from human or animal sources to plants were through soil and contaminated irrigation water supply (Jablasone *et al*., 2004). Moreover, during storage and transportation, manipulation of temperature and storage period could also contribute to the growth of *Salmonella*. Research findings done by Abushelaibi *et al*. (2003) demonstrated that with adequate refrigeration, the growth of *Salmonella* can be prevented. A minute amount of *Salmonella* could elevate to dangerous levels with increasing temperature and time.

From the results obtained in the present study, it proved that post-harvesting handlings such as the preparation steps by the hawker stalls are poor. Many hawkers are unaware of the importance of good sanitary procedures and the food safety levels. Most fresh fruits are washed with un-chlorinated water and all fruits are cut and blended using the same utensils without cleansing in between. According to Park *et al*. (2008), chlorinated or electrolyzed water can help to sanitize and decontaminate against foodborne pathogen of fresh fruits prior to consumption. Washing with running water can help to remove soil and dirt from the fresh fruits. However, through observation, we noticed that hawkers used the same bucket of stagnant water to rinse their fruits and utensils. Hence, the contaminants from the fruits are being kept and multiplied in the stagnant water where it exposed other uncontaminated fruits to *Salmonell*a. Besides, poor personal hygiene was observed during our sampling. These roadside vendors did not have proper attire and they did not use a sanitized glove. Measures to curb the lack of knowledge, education and exposure of these hawkers to proper food safety procedures are hence urgently needed.

Natural Food Hub (2000) computed that the levels of ascorbic acid (Vitamin C) in all fruits are different. Based on Adam *et al*. (2008), the minimum pH for growth varies with the acidulant within the fruit juices. The acidulants that contribute to the varied degrees of growth in *Salmonella* are acetic acid, hydrochloric acid, citric acid, malic acid, ascorbic acid and other subcomponents of acids. In this study, all fruit juices analyzed share a common acidulant which is the ascorbic acid. Referring to Table 5, the ascorbic acid of orange juice, starfruit juice, apple juice and watermelon juice were found to be at 53 mg/100 ml (Stella *et al*., 2000), 21 mg/100 ml (Nunes, 2008), 6 mg/100 ml (Lund *et al*., 1999) and 10 mg/100 ml (Stella *et al*., 2000) respectively.

Table 5. Ascorbic acid content and pH level in the fruit juices.

Fruit Juice	Ascorbic Acid Content (mg/100g)	pH	Reference
Orange	53	3.2-3.8	Stella *et al*., 2000; Natural Food Hub, 2000
Apple	6	3.8	Lund *et al*., 1999; Stella *et al*., 2000
Watermelon	10	5.5	Stella *et al*., 2000; Natural Food Hub, 2000
Starfruit/ Carambola	21	4.5	Nunes, 2008
Carrot	7.4	6.5	Gilbert *et al*., 2008

It is suggested that traces of ascorbic acid in fruit juices act synergistically to exert an inhibitory effect on microorganism. Conversely, Cortez *et al*. (2005) found that carrot juice has high amount of carotenoids, but very little amount of ascorbic acids. According to Abushelaibi *et al*. (2003), higher level of ascorbic acid and lower the pH of apple juice can inhibit or slow the growth of *Salmonella*. Even though apple demonstrated low pH values, but they have the lowest ascorbic acids content. The low pH in apple juice was contributed by high levels of malic acid. Hence from the comparison of data in our study, the higher prevalence of carrot juice to *Salmonella* spp. (60%) and *Salmonella* Typhi (40%) could be attributed to its lower ascorbic acid content. In addition, apple juice exhibited high levels of *Salmonella* Typhimurium (30%) due to the low levels of ascorbic acid. Moreover, it is clear that *Salmonella* Typhi survives better in higher pH condition, whereas *Salmonella* Typhimurium grows better in lower ascorbic acid condition.

Conclusion

The contamination of *Salmonella* in fresh fruit juice was a result of poor food handling. All fruit juice consumers of young and old are exposed to these potential health hazards. Proper temperature control and shorter storage period of fresh fruits are encouraged. Further research is needed to effectively determine the prevalence and source of contamination in fruit juices in various other parts of Malaysia to allow for a thorough risk assessment of *Salmonella* in fruit juices to be established so that corrective and preventive measures can be taken to ensure the quality and safety of fresh fruit juice in Malaysia.

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