International Food Research Journal 25(5): 2138-2142 (October 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Duplex PCR for the detection of *Salmonella* spp. and *Salmonella* Typhimurium in fresh coconut milk

Noorlis, A., *Nurul Ain, H. and Suwaibah, M.

Faculty of Applied Science, Universiti Teknologi MARA, 72000 Kuala Pilah, Negeri Sembilan Darul Khusus, Malaysia.

Article history

Received: 4 July 2017
Received in revised form: 27 September 2017
Accepted: 14 October 2017

Keywords

Salmonella spp.
Salmonella Typhimurium
Duplex MPN-PCR
Fresh coconut milk
Quantification
Food safety

Abstract

Fresh coconut milk is one of the main ingredients in most of the Malaysian cuisines. However, not many reports been published about *Salmonella* spp. in fresh coconut milk to date. The objective of this study was to investigate the preponderance and concentration of *Salmonella* spp. and *Salmonella* Typhimurium in fresh coconut milk in hypermarket and wet market level all around Senawang, Seremban and Kuala Pilah, Negeri Sembilan. A total of 120 samples of fresh coconut milk was obtained from hypermarkets and wet markets. The fresh coconut milk samples were collected from 1st March 2014 until 31st July 2014. Sampling area was done on the fresh coconut milk and proceeds with MPN-PCR methods. There is much difference between the prevalence of *Salmonella* spp. (74.2%) and *Salmonella* Typhimurium (29.2%) in hypermarket and wet market. The wet market at Seremban and Senawang was recorded as the highest prevalence of *Salmonella* spp. with 85%. Then, the presence of the *Salmonella* Typhimurium at the wet market have resulted 50.0%. The presence of *Salmonella* spp. (74.2%) is higher than *S.* Typhimurium (29.2%) which depends on the survival of the bacteria itself. *Salmonella* spp. has difference of pathogenicity to each other. *Salmonella* Enteritidis was not a selected host to survive, such as humans and many animals to grow but for the *Salmonella* Typhimurium, it is very selected to host and only certain animals like cattle, cow and farm

Introduction

Salmonella spp. was a common bacterium that infect human to caused bacteraemia, gastroenteritis, non-typhoidal salmonellosis and enteric fever (Pui *et al.,* 2011). Centers for Disease Control and Prevention (2013) reported Salmonellosis had recorded 1.2 million cases each year in United State and cause 450 deaths patients. It is a life-threatening scenario where it is also transmitted by the unhygienic food preparation area (Carrasco *et al*., 2012). In Malaysia, the three cases reported were caused by *Salmonella* spp. Every year, *Salmonellae* infected human and caused many illnesses and hospitalized cases.

A death occurred after consumed food in wedding ceremony in Kedah at 2013 followed by a 2 deaths cases were reported due to consumption of ready-to-eat foods at Kelantan in 2005 arose when the chicken was left for more than 4 hours outside at the open air and unclean utensils during food preparation (Tunung *et al.*, 2006; Embun, 2013). *Salmonella* spp. was transmitted by the faecal-oral route either by consumption of contaminated food or water, person-to-person contact, or from direct contact with infected animals (Jay *et al.,* 2003).

As mentioned in Malaysia Dietary Guideline (2008), the public awareness about fresh food products must include all of these criteria, such as unchanged colour of food, unpleasant odour and unchanged texture of food. The food industries have categorized the foodborne diseases into 3 types of foodborne hazard, such as biological, chemical and physical agent (Codex, 2015).

Coconut (Cocos nucifera) was also called as "The Tree of Life" in India, Philippine, Indonesia and Malaysia. Ancient India documented the application of coconut as Ayurvedic medicine in Sanskrit for more than 4000 years ago (Manisha and Shyamapada, 2011). Many treatments have been made to maintain the coconut milk against microbial spoilage. Nevertheless, it is discerned that the coconut milk product spoiled because of the lipid oxidation (Waisundara *et al*., 2007). The various nutrient content such as carbohydrate, sugars, fibre, saturated fat, protein, vitamin B_6, vitamin B_1 cause fresh coconut milk to be high potential carrier of *Salmonella* spp. (Carl, 1967; Uwubanwen *et al*., 2011).

According to the previous study, the most effective method to wisely detect the multiple

*Corresponding author.
Email: *ain_hasani@yahoo.com*

microorganisms in a single reaction and accurately identify the selected random gene or target gene in certain microorganism was by multiplex polymerase chain reaction (MPCR) or duplex polymerase chain reaction (Settani and Corsetti, 2007). Whereas, MPN-PCR was one of the quantitative methods to detect certain microorganisms in certain target gene.

The specific objective of this study was to investigate the preponderance and concentration of *Salmonella* spp. and *Salmonella* Typhimurium in fresh coconut milk in hypermarket and wet market level all around Senawang, Seremban and Kuala Pilah, Negeri Sembilan.

Materials and Methods

Sample preparation

A total of 120 samples were collected from wet markets and hypermarkets started on January to October 2015 in Kuala Pilah, Senawang and Seremban area in Negeri Sembilan, Malaysia. All samples were placed in a sterile and labelled ice container. The samples were analysed immediately on the day of sampling. The coordinate took plant sampling, latitude for the plant sampling was 2º47'41.79" N, and longitude 102º13'5.58" E.

Positive controls

Positive controls ATCC 13311 code for *Salmonella* Typhimurium and ATCC 13076 for *Salmonella* Enteritidis were obtained from the American Type Culture Collection (ATCC, Rockville, MD), United State of America. The strains of *Salmonella enterica* serotypes Enteritidis and Typhimurium were cultured onto tryptic soy agar (TSA; Merck, Darmstadt, Germany) and continued culture onto *Salmonella Shigella* agar (SSA; Merck, Darmstadt, Germany). Subsequently the bacteria were inoculated in 10ml of tryptic soy broth and incubated at 37°C for 24 hours.

Isolation and Pre-enrichment

Isolation method for *Salmonella* spp. was performed according to modified method of Wang *et al.* (2014) with a slightly modification. Ten millilitre samples were needed to pre-enrichment into 90 ml of Buffered Peptone Water (BPW, Merck; Darmstadt, Germany). All samples were incubated for 24 hours at 37°C.

Most probable number technique

A series of dilution of 10 ml was carried out up to 10^{-7} for each sample with tryptic soy broth and incubate for 24 hours at 37°C.The turbid tubes were subjected to duplex Polymerase Chain Reaction (PCR) for the detection of *Salmonella* spp. and *Salmonella* Typhimurium.

Table 1. Primer sequences used simultaneously in duplex PCR.

Target	Primer set name	Sequence (5'-3')	PCR product (bp)
Salmonella spp.	ST11	5'-GCCAACCATTGCTAAATTGGCGCA-3'	429
	ST15	5'-GGTAGAAATTCCCAGCGGGTACTGG-3'	
Salmonella Typhimurium	Fli15	5'-CGGTGTTGCCCAGGTTGGTAAT-3'	620
	Type04	5'-ACTGGTAAAGATGGCT-3'	

Extraction of total DNA

Total of DNA extractions were carried out from the turbid MPN tubes using cell-boiled method (Pui, 2011) with slightly modification. MPN turbid tubes were centrifuged for 1.5 ml at 13,000 x g for 3 minutes. The cell pellet was resuspend in 500 µl sterile distilled water and vortex vigorously. Then, the cell suspension was boiled for 10 minutes and cooled immediately at -20°C for 10 minutes. Repeat with centrifugation at 13,000 x g for 3 minutes. The clear supernatants were transferred into sterile new microcentrifuge tubes to be kept at -20°C freezer and later will be used as the DNA template in the duplex MPN-PCR.

Primers

A set of primers used in the duplex PCR was summarised in Table 1. The ST11/ST15 paired primer gene (429 bp) was pointed for the detection of *Salmonella* spp. and the primer set Fli15/Type04 was encoded as *fliC* gene were specifically used to detect *Salmonella* Typhimurium in the duplex PCR assay were synthesized by Next Gene Scientific Sdn. Bhd., Malaysia (Soumet, 1999; Pui, 2011).

PCR amplification

The duplex PCR amplification was performed on 96-well Thermal Cycler GTC (Clever Scientific Ltd., USA). Briefly, the duplex PCR amplification mixture 50 µl including 4 ul of DNA template contained 5x PCR buffer, 0.2 mM each deoxynucleoside triphosphate (dNTPs) mix, 1.5 U Taq DNA Polymerase and 4 ul DNA template solution and 1 µM of each primer pairs and 1.5mM of magnesium chloride ($MgCl_2$). The final volume of the reaction mixture was adjusted to 50µl using sterile distilled water. All the materials used in the PCR were

purchased from Promega (Interscience Sdn Bhd, Malaysia). A negative control containing sterile distilled water instead of the template DNA solution was included in each PCR assay. The thermo-cycling was programmed to 35 cycles : initial denaturation at 94°C for 2 minutes; denaturation at 94°C for 1 minute, primer annealing for 46°C for 1 minute and extension at 72°C for 7 minutes and maintenance at 4°C before electrophoresis. Five microliter of PCR products were mixed thoroughly with 1µl of RunSafe CSL (Nexbio Sdn Bhd) before loaded on a 1.0% (w/v) agarose gel and 0.5x TBE buffer. The gel was visualized using the Gel Documentation system (UVIDOC HD2, UVITEC, Bangkok, Thailand). A 100 bp ladder (Promega, Interscience Sdn Bhd, Malaysia) was used as a molecular size marker.

Table 2. Prevalence of *Salmonella* spp. and *Salmonella* Typhimurium in fresh coconut milk from market (MPN-PCR)

Market	Area	*Salmonella* spp.		*Salmonella* Typhimurium	
		No.[a]	%[b]	No.[a]	%[b]
Hypermarket	Seremban	15/20	75.0	10/20	50.0
	Senawang	14/20	70.0	3/20	15.0
	Kuala Pilah	15/20	75.0	1/10	5.0
Wet Market	Seremban	17/20	85.0	10/20	50.0
	Senawang	17/20	85.0	3/20	15.0
	Kuala Pilah	14/20	70.0	8/20	40.0
	Total	89/120	74.2	35/120	29.2

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

Results and Discussion

In Malaysia, fresh coconut milk is available in hypermarket and wet market. A total of 120 samples were analysed for the prevalence of *Salmonella* spp. and *Salmonella* Typhimurium in fresh coconut milk samples by using the specific primer for detection of *Salmonella* spp. (429bp) and *Salmonella* Typhimurium (620bp) respectively. *Salmonella* spp. and *Salmonella* Typhimurium were detected in hypermarket and wet market located around Seremban, Senawang and Kuala Pilah. Cross-contamination and recontamination of *Salmonella* spp. in fresh produce products have been recognized worldwide (Carraasco *et al.*, 2012). According to the previous study, non-sterile fresh coconut milk should be free from *Salmonella* spp. if handle in a clean environment and free contamination from mishandling of fresh produce (Carraasco *et al.,* 2012)

Table 2 was illustrated the highest prevalence of *Salmonella* spp. in wet market in Senawang (85.0%) and Seremban (85.0%) rather than hypermarket in Senawang (70.0%) and Seremban (75.0%) by using MPN-PCR method. For the hypermarket, the highest value of *Salmonella* spp. was at Seremban and Kuala Pilah areas were spotted for 75% appearance. This is because of the inappropriate store room and unhygienic condition in chiller that keep the ready-pack fresh coconut milk. Normally, fresh produce like fresh coconut milk was contaminated during production, harvest, processing, at retail levels or in the kitchen at home. Otherwise, the importance of washing procedure was one of the most studied to reduce the level of contamination (Carrasco *et al*., 2012; Diana *et al.*, 2012).

Salmonella Typhimurium showed the highest prevalence at Seremban for wet market (50.0%) and hypermarket (50.0%). As compared to many studies on *Salmonella* spp. detection in fruit juices, food and beverages, the isolation of *Salmonella* spp. in fresh coconut milk in this study is high (Radji *et al*., 2010; Diana *et al.*, 2012).

The highest value of the prevalence of *Salmonella* spp. was at Senawang and Seremban area in wet market was 85.0%. The lowest of prevalence *Salmonella* spp. in Kuala Pilah area with 70.0%. The high level prevalence of *Salmonella* spp. and *Salmonella* Typhimurium of the sterilized fresh coconut milk either with or without preservatives has high potential to support the growth of pathogens (Robert *et al.*, 2006).

As reported by Ministry of Health Malaysia (2017), all food preparation must follow the Food Safety and Quality Standard by Malaysia government. Food have been cooked must store in cold temperature (4°C) or at above 60°C. Normally, bacteria can multiply often in the temperature of danger zone (5°C to 62°C). Thus, the proper temperature storage which less than 5ºC shall be applied to avoid pathogens growth which is harmful to human (Ministry of Health, 2017).

Figure 1 indicated the highest presence of (MPN-PCR) overall for *Salmonella* spp. was stated at Seremban (32/40) and the lowest value at Kuala Pilah (29/40). The most high-pitched the presence of S. Typhimurium was stamp down by Seremban with 20/40 and the lowest value stated was at Senawang (6/40). The findings of *Salmonella* spp. were high for each area because of the survival of this bacterium can actively grow through host and non-host environments (Mollie *et al*., 2003).

Referring to Figure 2 and 3 showed the prevalence of *Salmonella* spp. (429bp) and *Salmonella* Typhimurium (620bp). As reported by Mollie et

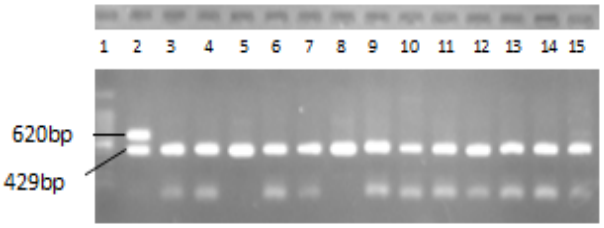

Figure 1. Gel electrophoresis image for identification of *Salmonella* spp. and *Salmonella* Typhimurium. Lane 1 100 bp ladder and lane 2 shows positive control (*Salmonella* spp. and *S.* Typhimurium). Lane 3 to lane 15 shows PCR amplicons specific to *Salmonella* spp. at 429 bp.

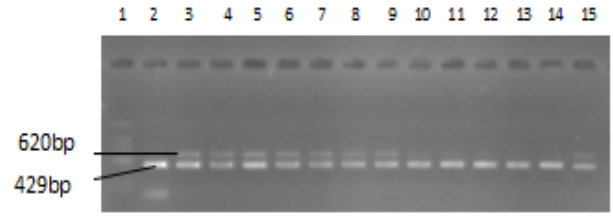

Figure 2. Gel electrophoresis image for identification of *Salmonella* spp. and *S.* Typhimurium. Lane 1 shows 100 bp ladder and Kuala Pilah Wet Market

al. (2003), *Salmonella s*pp. has high appearance on food because of the survival life cycle of *Salmonella* spp. which not fastidious pathogen to survive in host and non-host environments. *Salmonella* spp. can survive in mammals, reptiles, birds and insect. *Salmonella* spp. can survive in external environment with a minimum low nutrient support, wide range of temperature, and can live with or without oxygen (Winfield and Groisman, 2003). Therefore, the ability of *Salmonella* spp. live at vigorous growth of conditions, it showed that the reason *Salmonella* Enteritidis and *Salmonella* Typhimurium have high prevalence in wet market and hypermarket in Negeri Sembilan.

Conclusion

The prevalence of *Salmonella* spp. and *Salmonella* Typhimurium was high in fresh coconut milk and many serve as an agent of salmonellosis as an evident of this study. Thus, the coconut milk processing and storage shall be minimum at 5ºC in the chiller. Fresh coconut milk need to treat with heat treatment and have a good cleaning process of coconut machine to ensure the safety of food before passed on consumers.

Acknowledgements

This study was supported by Dana Pembudayaan Penyelidikan (project no. RAGS/2013/UiTM/SG 05/5) Ministry of Higher Education, Malaysia.

References

Baron, S. 1996. Medical Microbiology 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. Available from: *http://www.ncbi.nlm.nih.gov/books/NBK7627/*

Carl, P. S., Klaus, M., Venuso, C. B. and Colin, H. W. 1967. Coconut and *Salmonella* infection. Applied Microbiology 15(3): 471 – 475.

Carrasco, E., Andres, M. R. and Rosa, M. G. G. 2012. Review: Cross-contamination and recontamination by Salmonella in foods: A Review. Food Research International 45: 545 – 556.

CDC (Centers for Disease Control and Prevention). 2013. National *Salmonella* Surveillance Annual Report 2011. Atlanta, Georgia: US Department of Health and Human Services, CDC.

Codex. 2015. Retrieved on 10 September 2015 from Codex Alimentarius standard website: *www.codexalimentarius.net/gsfaonline/additives/details.html?id=129*

Diana, J. E., Pui, C. F. and Son, R. 2012. Enumeration of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in fruit juices. International Food Research Journal 19(1): 51 – 56.

Embun Majid. 2013. 'Ayam masak merah' contaminated. The Star Online. Retrieved from *http://www.thestar.com.my/news/nation/2013/10/02/ayam-masak-merah-contaminated-cook-who-made-the-dish-is-in-shock-say-relatives/*

Food Safety and Quality, Ministry of Health. 2015. Pemilihan dan Penyimpanan Makanan. Retrieved on August 6th, 2015 from Ministry of Health: *http://fsq.moh.gov.my/v4/index.php/component/k2/item/216-pemilihan-dan-penyimpanan-makanan.*

Food Safety and Quality, Ministry of Health. 2017. Panduan Kebersihan dan Kesihatan pada Makanan. Retrieved on April 12th, 2017 from Ministry of Health: *http://fsq.moh.gov.my/v5/ms/senarai-booklet-buku-kecil-berkaitan-keselamatan-makanan/*

Jay, L. S., Davos, D., Dundas, M., Frankish, E. and Lightfoot, D. 2003. Foodborne microorganisms of public health significance 6th ed, Ch. 8: *Salmonella,* p. 207-266. Sydney: Australian Institute of Food Science and Technology (NSW Branch).

Malaysian Dietary Guidelines. 2008. Key Message 13: Consume safe and clean foods and beverages. Retrieved on 10 October 2015 from Ministry of Health website: *www.moh.gov.my/images/gallery/Garispanduan/diet/km13.pdf*

Manisha, D. and Shyamapada, M. 2011. Coconut (*Cocos nucifera* L.: Arecaceae): In health promotion and disease prevention. Asian Pacific Journal of Tropical Medicine 214-247.

Ministry of Health Malaysia. 2014. "Keracunan Makanan (KRM) Di Bazaar Ramadan Taman Sri Muda, Shah Alam Selangor, Retrieved on September 8th, 2015.

From 1akses Malaysia: *http://1akses.malaysia.gov.my/OneGovSearchApps/widgetLoc/resultList.*

Mollie, D., Winfield and Eduardo, A. G. 2003. Mini Review: Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. Applied and Environmental Microbiology 69 (7): 3687-3694.

Parker, W. F. and Mee, B. J. 1982. Survival of Salmonella Adelaide and fecal coliforms in coarse sands of the swan coastal plain, Western Australia. Applied Environmental Microbiology 43: 981–986.

Pui, C. F., Wong, W. C., Chai, L. C., Lee, H. Y., Noorlis, A., Tuan Zainazor, T. C., Tang, J. Y. H., Mohamad Ghazali, F., Cheah, Y. K., Nakaguchi, Y., Nishibuchi, M. and Son Radu. 2011. Multiplex PCR for the concurrent detection and differentiation of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium. Tropical Medicine and Health 39(1): 9 –15.

Radji, M., Malik, A. and Widyasmara, A. 2010. Rapid detection of *Salmonella* in food and beverages samples by polymerase chain reaction. 2010. Malaysian Journal of Microbiology 6 (2): 166 – 170.

Roberts, T. A., Cordier, J. L., Gram, L., Tompkin, R. B., Pitt, J. I., Gorris, L. G. M. and Swanson, K. M. J. 2006. Microorganisms in food 6: Microbial ecology of food commodities. Chapter 9: Nuts, Oilseeds and Dry Legumes. International Commission on Microbiological Specification for Food (ICMFS), p. 458. United State: Springer Science & Business Media.

Settanni, L. and Corsetti, A. 2007. The use of multiplex PCR to detect and differentiate food- and beverage-associated microorganisms: A review. Journal of Microbiology Methods 69: 1-22.

Soumet, C., Ermel, G., Rose, V., Rose, N., Drouin, P., Salvat, G. and Colin, P. 1999. Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium and *Salmonella* Enteritidis strains from environmental swabs of poultry houses. Letters in Applied Microbiology 29: 1-6.

Tunung, R., Chai, L. C., Usha, M. R., Lesley, M. B., Cheah, Y. K., Patrick, G. B., *et al.* (2006). Incidence and characterization of *Salmonella* species in street food and clinical samples. Journal of Food Safety 27:345-361.

Uwubanmwen, I. O., Nwawe, C. N., Okere, R. A., Dada, M. and Eseigbe, M. 2011. Harnessing the potentials of the coconut palm in the Nigerian economy. World Journal of Agricultural Sciences 7(6): 684 – 691.

Waisundara, V. Y., Perera, C. O. and Barlow, P. J. 2007. Effect of different pre-treatments from fresh coconut kernels on some of the quality attributes of the coconut milk extracted. Food Chemistry 101: 771-777.

Wang H., Vikas S. G., Chorng, M. C., Narjol, G. E., Kari, A. I., Jie, Z., Rebecca, L. B., Andrew, P. J. and Thomas, S. H. 2014. Evaluation and comparison of rapid methods for the detection of *Salmonella* in naturally contaminated pine nuts using different pre enrichment media. Food Microbiology 46: 58 – 65.

Winfield, M. D. and Groisman, E. A. 2003. Minireview: role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli.* Applied and Environmental Microbiology 69 (7): 3687-3694.