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.**

**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 B₆, vitamin B₁ 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
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*.

**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 (MgCl₂). The final volume of the reaction mixture was adjusted to 50µl using sterile distilled water. All the materials used in the PCR were

<table>
<thead>
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<th>Table 1. Primer sequences used simultaneously in duplex PCR.</th>
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<td><strong>Target</strong></td>
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<td><em>Salmonella</em> spp.</td>
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<tr>
<td></td>
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<tr>
<td><em>Salmonella Typhimurium</em></td>
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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.

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 the prevalence of Salmonella spp. was at Seremban and Kuala Pilah area was 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 (Carraasco 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 Salmonella spp. (429bp) and Salmonella Typhimurium (620bp). As reported by 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

<table>
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<th>Market</th>
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<th>Salmonella spp.</th>
<th>Salmonella Typhimurium</th>
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<tr>
<td></td>
<td>No.¹</td>
<td>%²</td>
<td>No.¹</td>
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<td>70.0</td>
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<tr>
<td></td>
<td>Kuala Pilah</td>
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<td>75.0</td>
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<td></td>
<td>No.¹</td>
<td>%²</td>
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<tr>
<td></td>
<td>Seremban</td>
<td>17/20</td>
<td>85.0</td>
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<td>Senawang</td>
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<td>Kuala Pilah</td>
<td>14/20</td>
<td>70.0</td>
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Table 2. Prevalence of Salmonella spp. and Salmonella Typhimurium in fresh coconut milk from market (MPN-PCR)

¹Number of positive samples/number of samples examined
²Frequency (in %) of positive samples among the samples examined
Salmonella spp. 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 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.

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References


