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FULL PAPER

Could food or food contact surfaces be the favourable hideouts for *Listeria monocytogenes* in Perak, Malaysia?

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Abstract

Listeria monocytogenes is a causative agent of foodborne listeriosis which causes a high rate of hospitalisation (>90%) and death (20-30%) worldwide. Due to its ubiquitous nature and high resistance to a stressful environment, *L. monocytogenes* is able to multiply to a threatening level during food processing, distribution and storage, which then causes an immense case of foodborne disease outbreak. Hence, the control of *L. monocytogenes* is required at all stages in the food chain to prevent its occurrence in the final product. The present study aimed to determine the prevalence of *L. monocytogenes* in food as well as food contact surfaces from food processing plants and food service premises located in Perak, Malaysia. A total number of 170 food samples, including raw, minimally processed, processed and ready-to-eat food, as well as 152 samples from surfaces, including food-contact and non-food-contact, were collected and isolated on culture, and confirmation was done using polymerase chain reaction (PCR). A total of 26 food samples (15.29%) were positive for *L. monocytogenes,* with the highest prevalence found in processed and minimally processed food at 33.33% and 31.25%, respectively; following by raw and ready-to-eat food at 26.32% and 4.26% respectively. On the other hand, a higher prevalence of *L. monocytogenes* was detected from food-contact surfaces at 11.83% compared to non-food contact surfaces at 6.78%. These findings demonstrated the potential risk of contamination by *L. monocytogenes* in food and it might be due to the exposure of the bacteria on food processing surfaces. Thus, regular surveillance and strict assessment should be conducted by the local authorities to ensure the safety of the food consumption for residents in Perak.

1. Introduction

Listeria monocytogenes is a Gram-positive, non-sporeforming and facultative anaerobic bacteria (Farber and Peterkin, 1991; Liu, 2006). It is the most pathogenic species among the 19 *Listeria* genus members, to human and a range of other animals (Orsi and Wiedmann, 2016; Dojiad *et al*., 2018). Compared to other foodborne microbial pathogens such as *Salmonella* spp., *Escherichia coli* and *Campylobacter jejuni, L. monocytogenes* causes serious listeriosis foodborne outbreaks worldwide, especially in countries of United States of America, European Union, Australia, Nigeria and Asia (Hernandez-Millian and Payeras-Clfre, 2014; Al-Nabulsi *et al*., 2015; Effimia, 2015; Ng, 2018; Whitworth, 2018; Smith *et al*., 2019). Infected individuals, especially the vulnerable group of "YOPI": young, old, pregnant and immune-compromised, can lead to episodes of stillbirths, meningitis, septicaemia, gastroenteritis, fever and ultimately resulting in death (Franciosa *et al*., 2001; De Cesare *et al*., 2007; Silk *et al*., 2012).

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L. monocytogenes is able to adapt to extreme environments, such as surviving in a wide range of pH condition from 4.5 to 9.5; multiplying at low refrigeration temperature as -1°C and remaining viable in high salt condition at 40% w/v (Chasseignaux *et al*., 2002; Bucrieser *et al*., 2003; Liu, 2006; Meloni *et al*., 2009; Al-Nabulsi *et al*., 2015). Due to its durable characteristics, this extremophile grows easily on various food surfaces and forms biofilm: an aggregation of micro -colonies on the surfaces which enables substrate exchange, dissemination of metabolic products and elimination of toxic end products in order to sustain the growth of the bacteria communities (Donlan, 2001). The microbial biofilms immerse in a self-produced matrix of extracellular material, which consists of a conglomeration of different types of biopolymers known as extracellular polymeric substances (EPS's) (Donlan and Costerton, 2002). These structures enable a formation of strong adhesion of *Listeria* spp. to the surfaces and play a role in protecting the bacteria from antimicrobials invasion and shearing forces, as well as in tolerating the components of both the innate and adaptive immune response and resists phagocytosis. Thus, biofilms production is an important source of *L. monocytogenes* contamination that can lead to food spoilage or transmission of disease (Lundén *et al.*, 2000).

In Malaysia, several prevalence studies had been reported for the presence of *L. monocytogenes* in various foods such as meat, chicken offal, beef offal, beef patties, vegetables, salad and ready-to-eat food (Arumugaswamy *et al.*, 1994; Tang *et al*., 1994; Ponniah *et al*., 2010; Marian *et al*., 2012; Wong *et al.*, 2012; Kuan, Goh, Loo *et al.*, 2013; Kuan, Wong, Pui *et al.*, 2013; Marian *et al*., 2019). Contamination of the food could occur at all stages in the food chain especially at the surfaces of the food processing plant and during the distribution of the end products at the premises. This happened in the year 2015, when our Ministry of Agriculture and Food Industry seized the import of *L. monocytogenes* contaminated apples from Bidart Bros in Bakersfield, California (Malay Mail, 2015). The cause of contamination might be due to the occurrence of cross-contamination of the food in the farm with food contact surfaces of food processing plants or retail premises. Thus, this study aimed to investigate the occurrence of *L. monocytogenes* in food and on food contact surfaces in Perak, Malaysia by the culture-method and duplex-PCR (d-PCR) molecular method.

2. Materials and methods

2.1 Sample collection

From August 2018 to August 2019, a total number of 322 samples (170 food samples and 152 food contact surface samples) were randomly collected from six food premises, two food processing plants, four hypermarkets, a wet market and a night market in Perak, Malaysia. The types of samples collected are summarised in Table 1.

Table 1. The number of samples (shown in bracket) collected in this study. The types of collected samples consisted of food samples (raw, minimally processed, processed and ready-to-eat food) and swab samples from direct and indirect food contact surfaces

Source/Total	Samples Collected
Food (170)	
Raw Food (38)	Vegetables (23), Fresh meat and seafood (10), Bean sprouts (5)
Minimally Processed Food (33)	Minced meats (2), Pre-cut meats (5), Pre-cut fishes (6), Pre-cut vegetables (18), Bean curds (2)
Processed Food (6)	Quick-frozen meat products (4) Vacuum-packed meat products (2)
Ready-to-eat (RTE) Food (93)	Sandwiches (13), Salads (10), Fruits (12), Sushi rolls (6), Desserts (25), Cooked food (27)
Food Contact Surfaces (152)	
Direct Food Contact Surface (93)	Working benches for food processing Food processing machines (19), Cutting boards (7), Conveyor belts (9), Food preparation tools (mixing bowl, Surfaces of food product (11)
Indirect Food Contact Surface (59)	Trolleys (2), Display racks (9), Rack stands (6), Racks for intermediate storage (9), Pallets (5), Walls of cold storage environment (4), Cleaning equipment (wiping clothes)

2.1.1 Food samples

Food samples were purchased freshly from the hypermarkets, wet market and night market. Food from different categories was picked randomly from different stalls of each sampling site. The raw, minimally processed and processed foods were kept separately in cold storage boxes whereas ready-to-eat foods were kept under room temperature during the transportation back to the laboratory for analysis.

2.1.2 Swab samples of food contact surfaces

Swab samples from food contact surfaces were collected according to the protocol described by Public Health England (2017) with slight modifications. Individually packed sterilised cotton swab was pre-moistened with sterilised 0.85% (w/v) of saline solution (Merck, Germany). Then, a sterilised swab template of size 10 × 10 cm was placed on the tested area and was swabbed from left to right, up to down and at diagonal sides for 30 s. Rotation of the swab was performed during the collection process. The swabbed cotton was placed into a labelled tube containing 10 mL of 0.1% (w/v) peptone water (LAB M, United Kingdom). Collected samples were stored in a cold storage box during transportation to the laboratory. The samples isolation was then performed within 24 hrs.

2.2 Pre-enrichment, enrichment and purification steps

The detection and isolation of *L. monocytogenes* from the collected food samples was performed based on the procedure described by Kuan, Goh, Loo *et al.* (2013) and Kuan, Wong, Pui *et al.* (2013) with slight modifications. In brief, 25 g of food sample (from section 2.1.1) was placed in a sterile stomacher bag and homogenised with 225 mL of Listeria Enrichment Broth (LEB) (Merck, Germany) for 2 min using stomacher machine of BagMixer® 400P (Interscience, France). The suspension of 250 mL was then incubated for 4 hrs at 30°C, before adding the selective supplements agents: acriflavin, 10 mg/L, sodium nalidixate, 40 mg/L, cycloheximide 50 mg/L (Merck, Germany). Incubation was performed for 44 hrs at 30°C.

On the other hand, the collected swab sample of food contact surfaces (from section 2.1.2) that had immersed in 0.1% (w/v) peptone water was vortex for 3 min. A 1 mL portion of the suspension was then transferred and homogenised in 9 mL of LEB and incubated for 4 hrs at 30°C before the enrichment supplements were added. The samples were further incubated for 44 hrs at 30°C.

After 48 hrs of incubation, 0.1 mL of broth for all samples were spread plated on PALCAM agar (Oxoid, UK) and incubated for another 48 hrs at 30°C. Five presumptive colonies were picked from each PALCAM agar plate and sub-cultured onto Tryptic Soy Agar (TSA) (Merck, Germany). TSA agar was then incubated for 48 hrs at 30°C. This step was performed to purify the *Listeria* colonies before DNA extraction was performed.

2.3 Extraction of DNA

The boiled cell method was used to extract the DNA of the presumptive colonies from TSA plates, as described by Kuan *et al*. (2017). Briefly, one full loop of culture was scrapped from the TSA plate and resuspended in 200 µL of sterile distilled water. The suspension was then vortex prior to the boiling step at 100°C for 10 mins. The cell was then cooled at -20°C for 10 min before it was centrifuged at 13,400 *x g* for 3 min. The supernatant was subjected to PCR to identify the *Listeria* spp. and *L. monocytogenes* strains.

2.4 Duplex Polymerase Chain Reaction (d-PCR)

A total of 1,842 presumptive colonies were isolated and verified using duplex-polymerase chain reaction (d-PCR). d-PCR was carried out using two primer pairs: LI1 and U1, sequences as LI1-5' CTC CAT AAA CGT GAT CCT 3' and U1-5' CAG CMG CCG CGG CGG TAA TWC 3'; as well as LM1 and LM2, sequences as LM1-5' CCT AAG ACG CCA ATC GAA 3' and LM2-5' AAG CGC TTG CAA CTG CTC 3'. The first pair was a genus-specific primer for *Listeria* spp. which amplified at 16S rRNA gene with the size of 938 bp, whereas, the second pair was a species-specific primer for *L. monocytogenes* amplified at *hly*A gene with the size of 702 bp. Both primer pairs were synthesised by Apical Scientific Sdn. Bhd. d-PCR amplification was performed as described by Kuan, Goh, Loo *et al.* 2013 and Kuan, Wong, Pui *et al.* (2013) with slight modifications. d-PCR was conducted in a reaction mixture of 25 µL which contained 5 µL of 5X PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 0.2 µL of 10 mM deoxynucleoside triphosphate mix, 0.3 µL of 1.5U *Taq* DNA Polymerase, 0.5 µL of LI1 primer, 0.5 µL of U1 primer, 0.5 µL of LM1, 0.5 µL of LM2, 14.0 µL of sterile distilled water and 2.0 µL of DNA template (supernatant from the extraction of DNA). All reagents used in the PCR amplification were obtained from Promega (Research Instruments, USA). *L. monocytogenes* ATCC 19115 was used as a positive control for each PCR assay. The PCR conditions used was as such: initial denaturation at 94°C for 5 mins, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 2 mins, followed by a final extension step at 72°C for 7 mins. The thermal cycling reactions were performed using Thermal Cycler (Matrioux, Malaysia). Then, the PCR products were subjected to 1.5% agarose gel electrophoresis in 0.5X of Tris-Borate-EDTA (TBE) buffer at 100V for 45 min. The gel was then stained with 3X gel red and visualised under a gel imager (Bio-rad, USA). A 100 bp DNA ladder (Vivantis Technologies, Malaysia) was used as a DNA marker to estimate the size of amplified PCR products.

3. Results

3.1 Detection of Listeria monocytogenes in food and on food contact surfaces

The presence of *L. monocytogenes* was detected on PALCAM agar plate which exhibited colonies of grey-green colour with a black centre (Figure 1). The genus of *Listeria* was confirmed via PCR amplification of 16S rRNA which yielded 938 bp in size, while the presence of the species was confirmed via amplification of its haemolysin gene that encodes for *listeriolysin O* (*hlyA*) gene at 702 bp product (Wong *et al*., 2012) (Figure 2).

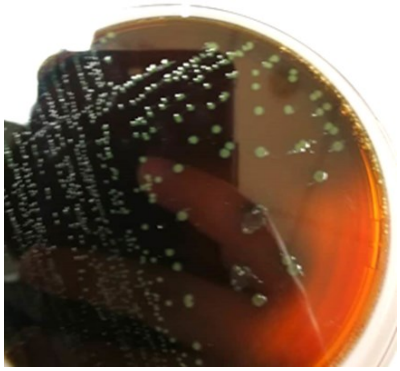

Figure 1. The growth of grey-green coloured colonies with black centre on PALCAM agar exhibited the presence of *L. monocytogenes*

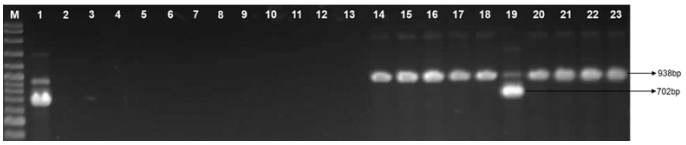

Figure 2. The image of 1.5% gel electrophoresis showed the PCR amplification of genes 16S rRNA (938 bp) and *hly*A (702 bp) for the detection of *Listeria spp*. and *Listeria monocytogenes*, respectively. Lane M: 100 bp DNA ladder (Vivantis Technologies, Malaysia); Lane 1: *L. monocytogenes* ATCC 19115 strain (positive control); Lane 2: Distilled water as template (non-template control); Lane 3 to 13: *Listeria* spp. negative samples; Lane 14 to 18: *Listeria* spp. positive samples; Lane 19: *L. monocytogenes* positive sample; Lane 20 to 23: *Listeria* spp. positive samples

3.2 Prevalence of Listeria monocytogenes in food and on food contact surfaces

Out of 170 food samples collected, 38 (22.35%) were raw food, 33 (19.41%) were minimally processed food, 6 (3.53%) were processed food and 93 (54.71%) were ready-to-eat food. Among 152 swab samples of contact surfaces collected, 93 (61.18%) were from direct food contact surfaces and 59 (38.82%) were from indirect food contact surfaces (Table 1). The prevalence of *Listeria*, as well as *L. monocytogenes* in the food samples and contact surfaces, were tabulated in Table 2. *Listeria* was found in 69 out of the 322 total samples (21.42%) collected and more than half of these samples were positive for *L. monocytogenes* (n=41/69, 59.42%).

Among the 26 *L. monocytogenes* positive food samples, processed food showed the highest prevalence at 33.33% (2 out 6 samples), followed by minimally processed food which accounted for 31.25% (10 out of 33 samples), raw food at 26.32% (10 out of 38 samples) and ready-to-eat (RTE) food at 4.26% (4 out of 93 samples). Nevertheless, *Listeria* was found in about 18.28% (17 out of 93 samples) in RTE food, which accounts for some attention on its presence. On the other hand, direct food contact surface was found to be more easily contaminated by *L. monocytogenes* with a prevalence of 11.83% (11 out of 93 samples) compared to indirect food contact surfaces, where these bacteria were detected at a prevalence of 6.78% (4 out of 59 samples).

Table 2. The prevalence of *Listeria* spp. and *L. monocytogenes* in food and on food contact surfaces.

Sources	No. of samples	No. (%) of *Listeria*	No. (%) of *L. monocytogenes*
Foods	170	49 (28.82)	26 (15.29)
Raw Food	38	14 (36.84)	10 (26.32)
Minimally	33	16 (48.48)	10 (31.25)
Processed Food	6	2 (33.33)	2 (33.33)
Ready-to-eat Food	93	17 (18.28)	4 (4.26)
Food Contact	152	20 (13.16)	15 (9.87)
Direct Food Contact	93	14 (15.05)	11 (11.83)
Indirect Food	59	6 (10.17)	4 (6.78)
Total	322	69 (21.43)	41 (12.73)

4. Discussion

Listeriosis is a life-threatening foodborne disease that is caused by the ingestion of food contaminated by *L. monocytogenes*. The questions ponder about the source of the contamination. It is postulated that the bacteria have come in contact at the food processing plant and start multiplying during the storage and at the retail level. In this study, it has been proved that both sources of food and food contact surfaces are at risk of getting exposed to *Listeria* contamination. Processed food, such as the frozen chicken slices and vacuum-packed smoked duck meat was observed to have the highest prevalence of *L. monocytogenes,* compared to minimally processed food, raw food and RTE food. On the other hand, direct food contact surfaces recorded a higher contamination level of *L. monocytogenes* than indirect food contact surfaces.

Processed food has a higher probability of being contaminated due to its long processing procedures needed. Before turning fresh food into food products, one or a combination of various processes, including washing, chopping, pasteurising, freezing, fermenting,

packaging and cooking are needed (USDA Agricultural Marketing Service, 2008). Despite the harsh treatment process involved, *L. monocytogenes* has the ability to strive for survival. Since most of the processed food is stored at low temperature in order to maintain its freshness, it may in turn create a favourable growth condition for the bacteria to proliferate. This observation was supported by a study conducted by Wong *et al*. (2012) who had reported that 22.33% of burger patties were detected positive for *L. monocytogenes.* In Assiut city of Egypt, El-Malek and colleagues (2010) had found a lower prevalence of *L. monocytogenes* detected in frozen meat and chicken samples. In addition, Marian *et al*. (2012) had reported that 33.3% of the burger samples collected from local wet markets, mini markets and supermarkets in Selangor, Malaysia were contaminated with *L. monocytogenes*. Thus, frozen processed meat products are a favourite reservoir for the growth of the foodborne pathogen.

Minimally processed food is another food material that has found to be easily contaminated by *L. monocytogenes*. According to the food classification tool (NOVA classification) developed by Monteiro *et al.* (2016), minimally processed food, including poultry, meat, seafood steaks, fillets, fresh-cut fruits and vegetables, as well as fresh or dried herbs such as mint or thyme, is a natural food product that undergoes mild processed without the addition of flavouring, salt and sugar. The mild processing steps involve sorting, washing, peeling, slicing, cutting, grinding and removing inedible parts of the fresh produce (Bansal *et al*., 2015). Again, the surfaces of grinders and some machinery in the process could be the potential sources of *Listeria* contamination due to the ideal temperature of the production area and improper cleaning of the complicated structure of machinery. In this study, *L. monocytogenes* was found in pre-cut vegetables (5/18), pre-cut meats (2/5), pre-cut fishes (2/6) and bean curds (1/2). The results were comparable with the study conducted in Japan and Turkey. Researchers from these two places had found the presence of *L. monocytogenes* in minced beef (12.2%) and ground beef (7.2%), as well as chicken meat samples (17.8%) from the retail premises (Inoue *et al*., 2000; Kalender, 2011).

On the other hand, raw food, either animal or plant sources, is natural or unprocessed foods that are obtained directly from farms (Poti *et al*., 2015; Monteiro *et al*., 2016). Although the percentage of *Listeria* spp. in the samples was less than 30%, its presence in 10 out of the 38 samples was a concern of listeriosis threat. Raw food might get contaminated from the soil, wastewater and faeces. Soil treated with artificial fertilizers creates a suitable growth environment for *Listeria* compared to soil treated with natural fertilizers (Szymczack *et al*., 2014). On the other hand, improper handling of wastewater management at the farms is often the case for the occurrence of cross-contamination (Lyautey *et al*., 2007). Thus, proper washing and cooking, preferably at 74^{o}C are encouraged to reduce the risk of contracting listeriosis.

In this study, 4.26% of the RTE food samples derived from fruits (1/12), sandwiches (1/13), desserts (1/25) and cooked food (1/27) were detected with *L. monocytogenes*. RTE vegetables, fruits and sandwiches are common sources for the sprouting of foodborne pathogens due to their zero heating processes and open-shelf storage in low refrigeration temperatures. Ponniah and colleagues (2010) had reported that 22.5% of RTE vegetables were positive for *L. monocytogenes*. Also, studies by Jamali *et al*. (2013), Leong *et al*. (2014) and Mureddu *et al*. (2014) had reported the presence of *L. monocytogenes* in RTE food samples at between 5.0 to 30.0%.

Elimination of *Listeria* poses a great challenge as it has the ability to strive for survival in harsh conditions. Hence, the strategy to mitigate the growth of the pathogen is to first detect the presence of *L. monocytogenes* in food processing plants and food contact surfaces before it reaches the final products. In this study, *Listeria* was found on the surfaces of the food processing machines such as food processing machines, conveyor belts, working benches for food processing and preparation, as well as on the surfaces of cutting boards. The highest prevalence of *L. monocytogenes* was found in food processing machines (6/19) such as roller bar, orientation and cooling machine. This observation was supported by Lundén *et al.* (2003), who stated that the complex machines in the processing lines were at high risk to be contaminated by *L. monocytogenes* due to irregular sanitisation of the food processing surfaces. Also, a low incidence of *L. monocytogenes* was found on the surfaces of processing tools used to produce sausages (Chevallier *et al*., 2006; Gounadaki *et al*., 2008). Following these incidences, the researchers in the US had revealed the presence of *L. monocytogenes* in the refrigerated food processing surfaces and equipment despite the regular cleaning and disinfecting (Carpentier and Cerf 2011; Hoelzer *et al.*, 2011). These studies had once again proved the high adaptability of *L. monocytogenes* in harsh environment.

Besides the direct food contact surfaces, non-direct food contact surfaces are a favourable choice for the growth of *L. monocytogenes*. In the food production area, floor and drains are retained in a cold and wet atmosphere, which create a notably favourable habitat

for *L. monocytogenes* to reside. This is seen in this study, whereby *Listeria* was detected in an area of display racks and cleaning cloths. This was also seen in the study by Leong *et al.* (2014), who had found a prevalence of 4.4% of environmental samples collected from 48 food business operators in the Republic of Ireland were positive for *L. monocytogenes*. On the other hand, contamination rate of *L. monocytogenes* on non-food contact surfaces such as floors, walls, drying rooms and steaming rooms in meat processing line were astonishingly high in the range between 11.0% to 25.0% (Thévenot *et al*., 2005; Mureddu *et al*., 2014).

In this study, the overall prevalence of *L. monocytogenes* detected in foods and on food contact surfaces from the food processing plant and food premises were low. Nevertheless, there is still a need for active surveillance in order to monitor the real scenario and to create awareness on the importance of cleanliness status in food and food contact surfaces from food processing plants and premises.

5. Conclusion

In conclusion, the overall prevalence of *L. monocytogenes* in food and food contact surfaces from food processing and food services environment are lower in Perak compared to studies in Selangor, Malaysia. However, this might pose a risk of listeriosis outbreak if further action is not taken as it will act as the route of transmission to the consumers. Food handlers, especially in the food industry and food premises have to perform robust surveillance, risk assessment and practice effective sanitary procedures in order to reduce the risk of growth of *L. monocytogenes*. It is important that efforts continue to understand the ability of the organism to survive and multiply under adverse conditions, and this knowledge can be used to design new control strategies.

Conflict of interest

The authors declare no conflict of interest.

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