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# Prevalence of *Listeria monocytogenes* in frozen burger patties in Malaysia

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**Abstract**

A total of 112 burger patties (35 beef burger patties, 39 chicken burger patties and 38 fish burger patties) which are commercially available at retail level were investigated for the presence and number of Listeria monocytogenes. These samples were analyzed using MPN-PCR method and conventional culturing methods. L. monocytogenes was detected in 33.3% of chicken burger patties, 22.9% of beef patties, and 10.5% of fish patty samples. From all contaminated raw burger patties, the estimated count of L. monocytogenes was ranged from 3 to 75 MPN/g. The results suggest that burger act as a potential source of listeriosis if the contaminated burger patty is consumed without adequate cooking. The risk associated with consumption of these samples was found to be high particularly for processed food at retail level in Malaysia. Therefore, food manufacturers play an important role in monitoring the manufacturing process and conduct a periodical surveillance on microbiological quality assessment on the processing plants. Besides, there is a need to increase awareness of consumers and food handlers to practice proper cooking of the burger patties before the point of consumption, to reduce the risk of listeria infection.



## Introduction

*Listeria monocytogenes* is a psychrotrophic foodborne pathogen which is widespread in the environment and in foods. It has caused a large number of foodborne outbreaks (Farber and Peterkin, 1991; McLauchlin, 1993). *L. monocytogenes* is able to adapt to a wide range of conditions such as refrigeration temperatures (2-4ºC), acidic environment, high salt concentration and within the host immune system. The high prevalence of *L. monocytogenes* in food in general, suggests that *L. monocytogenes* represents a potential threat to human health, especially to high risk groups such as immunocompromised patients, elderly, pregnant women and neonates. Listeriosis or *Listeria* infection is caused by eating food contaminated with *L. monocytogenes*. According to Meng and Doyle (1997), listeriosis is characterized by meningitis, encephalitis, and/or septicemia. It also causes cutaneous lesions and flu-like symptoms. The mortality rate for those hospitalized listeriosis patients is approximately 20%.

*L. monocytogenes* has been isolated from a variety of foods over the past fifteen years in Malaysia. Arumugaswamy *et al*. (1994) had reported the prevalence of *L. monocytogenes* in raw chicken (60%), raw beef (50%), fresh prawns (44%), leafy vegetables (22%), and ready to eat satay (26%); Hassan *et al*. (2001) carried out the same study on meat and fermented fish products, and found that the occurrence of *L. monocytogenes* in imported frozen beef was 75%, 30.4% in local beef samples and 12% in fermented fish. While a recent study done by Ponniah *et al*. (2010) had reported prevalence of *L. monocytogenes* in raw salad vegetables was as high as 22.5%. Although *L. monocytogenes* has been shown to be a frequent contaminant in foods, there has not been any reported case of listeriosis in Malaysia. Whether it is a case of true absence or failure in diagnosis is unknown.

Burgers are the most popular fast food worldwide, including Malaysia. Burger is one of the Malaysian's

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favorite western foods and can be easily found in high-end western restaurants, kiosks, night markets, street vendors and stalls in our community. However, unlike most western countries, homemade burger patties are uncommon in Malaysia. Here, ready-made burger patties, which are compressed and molded into disc-shape of one centimeter thick, are widely available in retail markets. These ready-made burger patties are made of either minced meat, minced chicken or fish paste with other common ingredients such as flour, oil, salt, pepper and some other preservatives. Ready-made burger patties are packed in a unit of ten pieces and sold as frozen product at retail level. Consumption of burger is in increasing popularity hence the biosafety of the burger patties is a great concern.

Quantification of *L. monocytogenes* had been done traditionally by most probable number (MPN) method, which consists of an estimation of density of viable organisms in a sample. However it is particularly useful for low levels of microbes that less than 10 to 100 MPN/g (Martin *et al*., 2004). Recently, combined MPN-PCR method had been used extensively by many researchers for enumeration of pathogens in foods and hence replace the conventional MPN method (Martin *et al*., 2004; Chai *et al*., 2007). The greatest advantage of PCR is its ability to amplify a defined region of DNA from a very complex starting template such as genomic DNA. In this study, the specific gene (hlyA) on L. monocytogenes that encodes for the listeriolysin O (LLO) is targeted in PCR detection of *L. monocytogenes*. The presence of *L. monocytogenes* is confirmed using primer pair LM1/LM2 which targeted on hlyA gene and produced amplicon at 702 bp. Another primer pair (U1/L1) targeted on the 16S rRNA of *Listeria* spp. to give amplicon at 938 bp. (Border *et al*., 1990).

The objective of this study is to detect the occurrence and numbers of *L. monocytogenes* in burger patties sold in retail outlets in Selangor. This study employed MPN-PCR method, MPN plating method and enrichment plating method for detection and enumeration of *L. monocytogenes*.

## Materials and Methods

*Sample preparation and pre-enrichment*

A total of 112 raw burger patties were purchased from two hypermarkets and a grocery shop in Selangor from June to October 2009. The burger patties chosen were representative of major brands in Malaysia. These samples were comprised of 35 beef patties, 39 chicken patties and 38 fish patties. All the samples were kept in an insulated ice box with ice packs and transported to the laboratory of analysis. The microbiological analysis of samples was initiated within 2 hours of sample collection.

The procedure was adapted from FDA-BAM Standard for detection of *Listeria* with modification according to the procedure by Ponniah *et al*. (2010). Briefly, 10 g of each sample was cut into small pieces and added into 90 mL of Listeria Enrichment Broth Base (LEB; Merck, Germany), then homogenized using a stomacher for 2 min. At the fourth hour of incubation at 30ºC, selective agents were added: acriflavin (final concentration: 10 mg/L); sodium nalidixate (final concentration: 40 mg/L); and cycloheximide (final concentration: 50 mg/L) (Sigma, USA).

*Detection and enumeration of L. monocytogenes*

Two successive dilutions were prepared using LEB (Merck). A total of 0.1 mL aliquot from each dilution was spread evenly on PALCAM Listeria-Selective agar (PALCAM; Merck, Germany). Inoculated PALCAM agar was incubated at 30˚C for 48 h. Any suspected colonies that grown on the agar plate were inoculated onto Tryptic Soy Agar (TSA; Merck, Germany) for purification and further confirmation by PCR assay.

For three-tubes MPN method, 1 ml aliquot from each dilution was transferred into triplicate MPN tubes, and then incubated at 30ºC for 48 h. The content of each tube was checked for turbidity after 2 days of incubation. All of the turbid MPN tubes after 48 h incubation were streaked on PALCAM agar (Merck) by using a loop and incubated at 30˚C for another 48 h, before subjected to DNA extraction and further PCR assay. Any suspected colonies that grown on the agar plate were inoculated onto TSA agar slant (Merck) for purification and confirmed by PCR assay.

*Genomic DNA preparation*

Genomic DNA was prepared by boiled-cell method (Tang *et al*., 2009; Lee *et al*., 2009; Noorlis *et al*., 2011) with modification. A total of 500 µL of aliquot from each MPN tube was subjected to centrifugation at 10,000 x g for 5 min to pellet the microorganisms. The pellet was resuspended in 500 µL of sterile TE buffer (pH8.0). The mixture was vortexed and boiled for 10 min. After boiling, the content was cooled at -20˚C immediately for 10 min before it was centrifuged at 10,000 x g for 10 min. The supernatant was obtained for use as the template DNA solution in PCR assay.

Table 1. Sequences of oligonucleotide primers used to target specific genes in *L. monocytogenes* and their respective amplicons, adapted from Border *et al*. (1990)

| Primer | Sequence (5' - 3') | Target region | Amplicon |
|---|---|---|---|
| LM1 | CCTAAGACGCCAATCGAA | *hlyA* | 702 bp |
| LM2 | AAGCGCTTGCAACTGCTC | | |
| U1 | CAGCMGCCGCGGTAATWC | 16*S* rRNA | 938 bp |
| LI1 | CTCCATAAAGGTGACCCT | | |

* M denotes A or C; W denotes A or T

*PCR assay*

The PCR amplification was performed in 25 µL of a reaction mixture containing 5 µL of 5X PCR buffer, 1.5 mM $MgCl_2$, 0.08 mM dNTP mix, 1.5 U Taq Polymerase, 10 pM of each primers (as listed in Table 1) and 2 µL of template DNA solution. All reagents were purchased from Promega, USA, and primers used were synthesized by Invitrogen. Thermal cycling was performed with the Veriti 96-Well Thermal Cycler (Applied Biosystems) using the following condition: initial denaturation at 94˚C for 5 min; 30 cycles of denaturation at 94˚C (30 s), annealing at 53˚C (1 min) and elongation at 72˚C (2 min); followed by a final extension at 72˚C for 7 min. DNA extracted from the *L. monocytogenes* ATCC 19155 was used as the positive control in each PCR assay. The PCR products were analyzed by size fractionation of DNA through 1.0% (w/v) of an agarose gel which was prepared by 0.5X TBE buffer. A total of 5 µL of amplified PCR product was loaded on an agarose gel and run at 100 V for 30 min. A 100 bp DNA-molecular ladder (Promega, USA) was included in the gel. The gel was finally stained with ethidium bromide and visualized under UV light using Gel Documentation System

## Results and Discussion

The prevalence of *L. monocytogenes* in different type of burger patties by using different detection and enumeration methods are summarized in Table 2. In general, the prevalence of *L. monocytogenes* in the burger patties examined was 22.32%, based on MPN-PCR assay. A representative of gel electrophoresis image of PCR result for *L. monocytogenes* from a MPN turbid tube was shown in Figure 1.

The high prevalence of *L. monocytogenes* in retail burger patties as revealed in current study is not surprising, as *L. monocytogenes* are well-documented for its high survivability under environmental stresses, such as high acidic and low temperature (Pal *et al*., 2008; Cunningham *et al*., 2009). Occurrence of *L. monocytogenes* in food products can be due to the widespread nature of *L. monocytogenes* in the environment, in which animals tend to carry and harbor the pathogen especially on hides (Rivera-Betancourt *et al*., 2004). During the processes of slaughtering, evisceration and sample preparation, cross contamination might occur and hence the raw meat is in great opportunity to become the host for the bacterium. Moreover, Rhoades *et al*. (2009) argued that extra handling and ingredients during processing of meat products give rise to the Listeria contamination, as shown in their survey, processed beef burger was observed to be more heavily contaminated than that of minced beef. It is reasonable to say that addition of preservatives and spices during the processing of burger patties and freezing during transportation and retailing are not sufficient to deactivate all *L. monocytogenes* that might presence in the raw meat at the initial step.

Table 2. Prevalence of *L. monocytogenes* in burger samples by using different detection and enumeration method

| Burger types | n | Enrichment plating | | MPN plating | | MPN-PCR | |
|---|---|---|---|---|---|---|---|
| | | n* | % | n* | % | n* | % |
| Beef | 35 | 7 | 20.0 | 6 | 17.1 | 8 | **22.9** |
| Chicken | 39 | 6 | 15.4 | 7 | 18.0 | 13 | **33.3** |
| Fish | 38 | 0 | 0 | 0 | 0 | 4 | **10.5** |
| Total | 112 | 13 | **11.61** | 13 | **11.61** | 25 | **22.32** |

n* = number of sample contaminated with *L. monocytogenes*

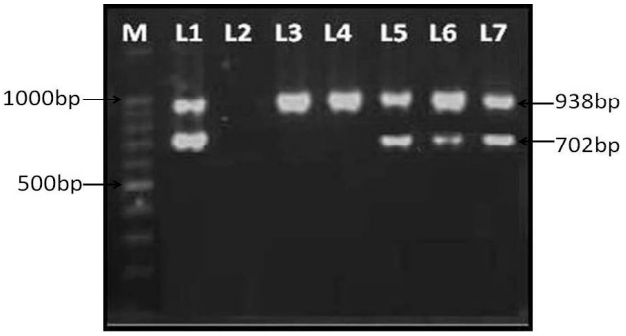


Figure 1. Electrophoretic analysis of 16S rRNA of *Listeria* spp. (938 bp) and *hlyA* gene of *L. monocytogenes* (702 bp). Lane M = 100-bp DNA ladder; L1 = Positive control of *L. monocytogenes* that shows PCR products at 702 bp and 938 bp; L2 = Distilled water as negative control of the PCR reaction; L3 – L4 = representatives of *Listeria* spp. from the samples; L5 – L7 = representatives of *L. monocytogenes* from samples

Among three different types of burger patties investigated, the highest prevalence of *L. monocytogenes* was found in chicken patties (33.3%), followed by beef patties (22.9%), and fish patties (10.5%). The prevalence of *L. monocytogenes* in chicken patties was found to be higher than that of beef. This finding is partly supported by previous studies done by other researchers saying that poultry products are more commonly contaminated with *L. monocytogenes* than beef products (Fantelli and Stephan, 2001; Miettinen *et al*., 2001). But somehow, previous studies do not focus on burger patties; therefore, this is an important assessment of biosafety

of one of the processed meats in Malaysia.

An interesting finding from this study is that, the fish burger patties harbored the least *L. monocytogenes* (10.5%). This is in contradiction with the vast literature (Brett *et al*., 1998; Ericsson and Stalhandske, 1997), pointing that fish and seafood such as cold smoked salmon, smoked roe, shrimps and mussels are very common to be recognized as a source for listeriosis at Western countries. For this, the joint WHO/FAO (2004) claimed that the main reason for the contradiction shall be the geographical differences. They stated that the prevalence of *L. monocytogenes* is considered to be much lower in fish products harvested from tropical waters than those derived from temperate waters. Malaysia is a tropical country and therefore, it is acceptable to say that the prevalence of *L. monocytogenes* in fish and seafood product is comparatively lower than those found at Western countries.

Density of *L. monocytogenes* present in burger patty samples was calculated using the data obtained from MPN-PCR method, in MPN/g. For the 25 samples that turned out positive for *L. monocytogenes*, the estimated count ranged from 3 to 75 MPN/g (results not shown). The highest count for *L. monocytogenes* was found in beef patties (75 MPN/g). Density of *L. monocytogenes* in fish patties was the lowest among all types of burger patty, with a minimum of 3 MPN/g and maximum 12 MPN/g. The maximum number of *L. monocytogenes* being detected in chicken patties was 43 MPN/g. *L. monocytogenes* had been detected in all types of burger patty. In fact, contaminated processed meat product is an indication of microbiological quality and hygienic status of a manufacturing plant. Kornacki and Gulter (2007) had reported that *L. monocytogenes* is highly occurred in ready to eat (RTE) meat processing facilities within North America and Europe, especially beef processing plant (28-92%), poultry processing plant (13.3%) and fish processing plant (12.8%). Besides the ability of *L. monocytogenes* to grow at low temperature, other factors that contribute to the persistence of *L. monocytogenes* in food processing environments are its biofilm forming abilities and sanitizer resistance on various food contact surfaces (Romanova *et al*., 2002; Di Bonaventura *et al*., 2008). However, before we can pinpoint the ultimate contamination source, an intensive on-site investigation on microbiological quality is essential, especially on the surface of processing equipments and facilities that have direct contact with the meat products, such as conveyor belt, chopping machine and packaging area, where biofilm may exist (Pui *et al*., 2011).

This finding suggests a relatively low concentration of *L. monocytogenes* in burger patties. Nonetheless, when exposed to temperature abuse condition and given enough time, *L. monocytogenes* will be able to multiply exponentially to a level where high risk groups are particularly threatened upon consumption of the undercooked burger patty (Hoan-Jen *et al*., 2007). Especially the emergence of hypermarkets in Malaysia and its convenience had contributed to the changes in shopping behavior of Malaysians. Nowadays, consumers tend to shop for more than hours for their household groceries, foods and personal care items at one time. Therefore, a temperature abuse condition that gives chances for bacteria multiplication might be created. On the other hand, there are a lot of well known street burger stalls in Malaysia. As observed from some burger stalls, during peak hour, the hawkers cooked the burger patties in rush. In a study done by Stephens *et al*. (1994) regarding the thermal inactivation of *L. monocytogenes* over a temperature range, the viable cell number of *L. monocytogenes* was reduced from log 9 to log 2 at 64ºC in 100 s. However, if the cooking is inadequate, the bacterium will survive and remain active to initiate a sequential of *listeria* infection symptoms in an individual once the bacterium enter and colonize the gastrointestinal tract.

When comparing the results of MPN-PCR to common culturing methods (direct plating and enrichment plating), the prevalence of *L. monocytogenes* found by both plating methods was 11.61%. The common culturing methods used in this study failed to detect all PCR-positive samples and this might suggest false negative result by using conventional plating method. In this study, the combined MPN-PCR method can detect at higher sensitivity compared to other methods. This was supported by previous studies (Shearer *et al*., 2001; Aznar and Alarcon, 2002). In their studies, more samples turned up positive when detection of pathogen in contaminated food samples was done by PCR-based method. In fact, Hayes *et al*. (1992) showed that NGFIS method (one of the culture dependant methods) only gave positive result for 74% of foods that was contaminated with *L. monocytogenes*, suggesting an unacceptable level of detection, when deaths may result if the pathogen is present but not being detected. Apart from that, enrichment methods do not account for recovery of sublethally injured cells that may present as a result of heating, freezing or acidification of foods (Buchanan, 1990). These manufacturing processes render the bacteria non-culturable, subsequently cause these bacteria neither form colonies under selective pressure during recovery, nor grow on the selective agar, hence turn

out as negative result upon incubation.

In conclusion, *L. monocytogenes* had been detected in burger patties. Since it is one of the most popular processed meat products in Malaysia, appraisal on the risk of infection after consumption of contaminated burger patties in the country is a necessity. Despite of this, the findings of this study is essentially important to be served as baseline data in risk estimation for the prediction of human listeriosis and further provide useful information for the microbial risk assessment of *L. monocytogenes* in Malaysian foods. It is recommended to carry out further study on characterization of *L. monocytogenes* isolates to reveal their genetic diversity which play important role in determining the virulence and pathogenicity of the strains.

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