

Research Paper

Prevalence and quantification of *Listeria monocytogenes* in beef offal at retail level in Selangor, Malaysia

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Abstract

A total of 63 beef offal samples (beef liver = 16; beef lung = 14; beef intestine = 9; beef tripe = 15; beef spleen = 9) from three wet markets (A, B, and C) in Selangor, Malaysia were examined for the prevalence and microbial load of *Listeria monocytogenes*. A combination of the most probable number and polymerase chain reaction (MPN-PCR) method was employed in this study. It was found that *L. monocytogenes* detected in 33.33% of the beef offal samples. The prevalence of *L. monocytogenes* in beef offal purchased from wet markets A, B, and C were 22.73%, 37.50% and 41.18% respectively. The density of *L. monocytogenes* in all the samples ranged from < 3 up to > 2,400 MPN/g. The findings in this study indicate that beef offal can be a potential vehicle of foodborne listeriosis.

Key words: *Listeria monocytogenes*, most probable number (MPN), polymerase chain reaction (PCR), beef offal, prevalence.

Introduction

Listeria monocytogenes is a Gram-positive, facultative anaerobic, non-spore forming foodborne pathogen which can cause a severe illness of listeriosis. Although listeriosis is relatively rare, it accounts for high fatality rate (30%) especially in YOPI (young, old, pregnant, immunodeficient) groups (Broome *et al.*, 1990; Berche 2005; Painter and Slutsker, 2007). This ubiquitous bacterium is widespread in the environment. In the last two decades, *L. monocytogenes* has caused in a high number of listeriosis outbreaks throughout the world and it was associated with various foods, including chocolate milk in United States of America, butter in Finland and cheese in Japan (Dalton *et al.*, 1997; Lyytikäinen *et al.*, 2001; Makino *et al.*, 2005). So far, listeriosis outbreak has not been reported in Malaysia. Previous studies have reported that *L. monocytogenes* has been found in poultry, seafood and vegetables in Malaysia over the past 15 years (Arumugaswamy *et al.*, 1994;

Endang *et al.*, 2003; Jeyaletchumi *et al.*, 2010b). Therefore, listeriosis might occur in Malaysia but deemed to be undetected due to the difficulty in identification *L. monocytogenes* which is closely similar with other *Listeria* spp. in term of morphology and biochemical properties (Liu 2006).

Internal organs of animal such as liver, spleen, lung, tripe and intestine can be the sources of *L. monocytogenes*. Arumugaswamy *et al.* (1994) reported that higher prevalence (63%) of *L. monocytogenes* was detected in chicken parts, liver and gizzard in Malaysia. The safety level of *L. monocytogenes* in beef offal is a great concern due to the eating habits of Malaysians. In Malaysia, beef offal is very popular as fried or in soups and almost all of the parts of the beef can be eaten. It can be easily obtained from wet markets with a cheap price. However, the hygienic conditions of these wet markets are poor which can increase the cross contamination rate and contributing to higher prevalence of *L. monocytogenes*.

Conventional methods for the detection of *L. monocytogenes* involve selective enrichment with subsequent selective plating, followed by serological confirmation and/or biochemical screening are considered as time consuming process (Jeyaletchumi *et al.*, 2010a). In this study, most probable number (MPN) method together with polymerase chain reaction (PCR) method was used for the quantitative detection of *L. monocytogenes* in beef offal samples. MPN-PCR has been proven to be a rapid method with higher accuracy in the detection of species specific genes of foodborne pathogens (Chai *et al.*, 2007; Su and Liu, 2007).

Although many studies have been carried out on the *L. monocytogenes* in different food products, there is limited data available on the prevalence of *L. monocytogenes* in animal offal. This study is aimed to determine the prevalence and microbial load of *L. monocytogenes* in beef offal by using the MPN-PCR method to obtain more information for the food safety risk assessment analysis thereby raise awareness among the consumers on the consumption of this type of food.

Materials and Methods

Sample collection

From December 2010 to February 2011, a total of 63 beef offal samples were randomly purchased from wet market A, B, and C respectively in Selangor, Malaysia. During sampling, all the samples were transported to the laboratory in an ice box and analyzed immediately.

Detection and enumeration of *L. monocytogenes* by MPN-PCR method

A 10 g of beef offal sample was aseptically weighed and added to 90 mL of *Listeria* Enrichment Broth (LEB, Merck) in stomacher bag and homogenized using stomacher for 2 min. The pre-enriched bacterial culture was then incubated at 30 °C for 4 h. Then, the selective agents (acriflavin, 10 mg/L; sodium nalidixate, 40 mg/L; cycloheximide 50 mg/L) were added. For three-tube MPN analysis, 1 mL of the 10-fold, 100-fold and 1000-fold dilutions of the enriched bacteria culture were incubated for another 44 h at 30 °C, then 0.1 mL was taken from each tube and plated on PALCAM *Listeria*-selective agar (Merck) before incubated for 48 h at 30 °C. At least five presumptive colonies (grey-green colonies with black zones) were picked and plated onto Tryptic Soy Agar (TSA; Difco™) for purification before undergoing confirmatory tests.

The turbid MPN tubes and presumptive colonies on TSA agar were subjected to DNA extraction by using boiled cell method (Chai *et al.*, 2007; Jeyaletchumi *et al.*, 2010b). A 500 µL portion of each broth was centrifuged at 13,400 g for 1 min, and the pellet was resuspended in 500 µL of sterile distilled water. The mixture was homogenized by using vortex and boiled for 10 min. Then, the cell suspension was cooled at -20 °C for 10 min before it was

centrifuged at 13,400 g for 3 min. The supernatant was used as the DNA template solution in the PCR. The primer pairs with the sequences of 5'CTC CAT AAA GGT GAC CCT 3' and 5'CAG CMG CCG CGG TAA TWC 3' was designed to specifically amplify a 938 bp fragment of the 16S rRNA gene for the detection of *Listeria* genus. Another set of primer pairs with the sequences of 5'CCT AAG ACG CCA ATC GAA 3' and 5'AAG CGC TTG CAA CTG CTC 3' was designed to amplify a 702 bp fragment of the listeriolysin O (LLO) gene for the detection of *L. monocytogenes*. All the primers were obtained from Bio-Diagnostics Sdn Bhd, Invitrogen Corporation.

The PCR amplification was performed in 25 µL reaction mixtures containing 5 µL of 5 x PCR buffer, 1.5 µL of 25 mM MgCl₂, 0.2 µL of 10 mM of deoxynucleoside triphosphate mix, 0.3 µL (1.5U) of Taq DNA Polymerase, 0.5 µL of each primers, 14 µL of sterile distilled water and 2 µL of DNA template. All the PCR reagents were purchased from Promega, Research Instruments, USA. Thermal cycling was carried out in Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) by using the thermocycler conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min, and extension at 72 °C for 2 min, and followed by a final extension at 72 °C for 7 min.

For visualization of PCR products, 3 µL of amplified PCR products was loaded in 1.0% agarose gel and run at 100 V for 28 min. Then, the agarose gel was stained with 0.1 µg mL⁻¹ of ethidium bromide and viewed using the Gel Documentation System (SynGene). A DNA-molecular ladder (100 bp ladder) (Vivantis Technologies, Selangor, Malaysia) was included in each gel.

Results

A total of 63 beef offal samples were examined for prevalence and microbial load of *L. monocytogenes* by using MPN-PCR method. The prevalence of *L. monocytogenes* in beef offal was 33.33% (21 positive samples out of 63 samples). Overall, the occurrence of *L. monocytogenes* was highest in beef lung (50.00%) followed by beef tripe (46.67%), beef liver (25.00%), and beef intestine (22.22%). There are zero occurrences of *L. monocytogenes* in beef spleen sample analyzed. The prevalence of *L. monocytogenes* in beef offal samples was tabulated in Table 1.

In this study, the target genes specific for *L. monocytogenes* produced PCR products of size 702 bp and 938 bp respectively. Figure 1 shows a representative PCR amplification of the 16S rRNA and LLO genes for identification of *Listeria* genus and *L. monocytogenes* respectively. Apart from enumeration of the prevalence of *L. monocytogenes* in beef offal samples, microbial load (MPN/g) of *L. monocytogenes* was also calculated. From the Table 2, the maximum numbers of *L. monocytogenes* in beef offal collected from wet market A, B, and C were

Table 1 - Prevalence of *L. monocytogenes* in beef offal samples purchased from Wet Market A, B, and C.

Beef offal	Wet market A			Wet market B			Wet market C			Total		
	n ^a	PCR positive	% ^c	n ^a	PCR positive	% ^c	n ^a	PCR positive	% ^c	n ^a	PCR positive	% ^c
Liver	5	0	0.00	5	3	60.00	6	1	16.67	16	4	25.00
Lung	4	1	25.00	4	3	75.00	6	4	66.67	14	8	50.00
Intestine	4	2	50.00	5	0	0.00	b ₋	b ₋	b ₋	9	2	22.22
Tripe	5	2	40.00	5	3	60.00	5	2	40.00	15	7	46.67
Spleen	4	0	0.00	5	0	0.00	b ₋	b ₋	b ₋	9	0	0.00
Total	22	5	22.73	24	9	37.50	17	7	41.18	63	21	33.33

^an = Number of sample.
^b(-) = Sample not available.
^c(%) = Percentage.

Table 2 - Microbial load of *L. monocytogenes* (MPN/g) in beef offal samples purchased from Wet Market A, B, and C.

Beef offal	Wet Market A			Wet Market B			Wet Market C		
	Min ^a	Med ^b	Max ^c	Min ^a	Med ^b	Max ^c	Min ^a	Med ^b	Max ^c
Liver	< 3	< 3	< 3	< 3	6.2	> 2,400	< 3	< 3	14
Lung	< 3	< 3	3.6	< 3	< 3	3	< 3	< 3	9.3
Intestine	< 3	< 3	6	< 3	< 3	< 3	^d ₋	-	-
Tripe	< 3	< 3	9.1	< 3	3.6	23	< 3	< 3	3.0
Spleen	< 3	< 3	< 3	< 3	< 3	< 3	-	-	-
Average	< 3	< 3	9.1	< 3	< 3	> 2,400	< 3	< 3	14

^aMin = Minimum MPN/g value.
^bMed = Median MPN/g value.
^cMax = Maximum MPN/g value.
^d₋ = Sample not available.



Figure 1 - Representative PCR amplification of the 16S rRNA and LLO genes for identification of *Listeria* spp. (genus) (938 bp) and *Listeria monocytogenes* (702 bp). Lane M shows the 100 bp DNA marker. Lane 5 and 6 show negative and positive control respectively. Lane 1 to 3 and lane 7 to 12 show *L. monocytogenes* positive samples whereas Lane 4 shows *L. monocytogenes* negative sample.

9.1 MPN/g, > 2,400 MPN/g, and 14 MPN/g respectively. Microbial load of *L. monocytogenes* in beef offal samples was summarized in Table 2.

Discussion

It was not surprising that *L. monocytogenes* was detected in the beef offal as slaughtered animals are recog-

nized as reservoirs of foodborne pathogens (Mead, 2007). From Table 1, it showed that prevalence level of *L. monocytogenes* in beef offal may vary from low to high. This is mainly due to the different handling practices carried out in those wet markets. Generally, contamination can happen in many ways such as the use of unhygienic containers during transportation and distribution, improper food handling practices, cross-contamination from other contaminated foods or from infected field workers at the market (Chai *et al.*, 2007). Other than hygiene problems, storage temperature is also known to be closely related with the distribution of foodborne pathogens (Su and Liu, 2007). Storage of beef offal under refrigeration conditions is limited in controlling the initial microbial load of *L. monocytogenes*. Due to the psychrotrophic characteristic, they can remain viable and proliferate in the cold conditions. Therefore, it is reasonable to believe that the longer holding time for the beef offal to be sold out, the higher the microbial load of *L. monocytogenes* in beef offal.

The PCR technique which employs an enzyme and oligonucleotide primers was found to be simple, rapid, less laborious, and more reliable as compared to the conventional plating method. PCR protocol designed for this study

is species specific and highly sensitive, which works based on virulent gene, LLO gene that present only in *L. monocytogenes* (Mengaud *et al.*, 1988). The primers used were specifically designed for detection of *L. monocytogenes* as they only amplified a 702 bp region in the LLO gene (Aznar and Alarcón, 2003).

The presence of *L. monocytogenes* in beef offal samples analyzed in this study indicated that *L. monocytogenes* can act as possible vehicles of infection for foodborne listeriosis. Unhygienic practices during handling, distribution, and storage are believed to worsen this scenario. Although foodborne outbreak of listeriosis in Malaysia is rare, it does not mean that listeriosis will not occur since studies showed that *L. monocytogenes* had been isolated from wide range of foods. *Listeria* infection is rarely reported as it is not among the more commonly notifiable bacterial infections such as *Salmonella* and *Escherichia* infection. Besides, epidemiology of listeriosis is being complicated and symptoms such as flu-like illness or gastroenteritis will cause the incidence of listeriosis become more difficult to be identified (Bortolussi 2008). Thus, high risk groups such as pregnant women, elderly people and immune-compromised patients advised to avoid consume any partially or undercooked beef offal because inadequate heat treatment is unable to completely destroy *L. monocytogenes* and other foodborne pathogens present. Further studies to understand the growth and sources of contamination of *L. monocytogenes* in beef offal during slaughtering, distribution and storage can be carried out, in order to develop a food safety risk assessment model to estimate the risks of listeriosis associated with beef offal.

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