

## Characterization of *Listeria monocytogenes* Isolated from A Fresh Food Factory

### (Karakterisasi *Listeria monocytogenes* yang Diisolasi dari Pabrik Makanan Segar)

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**Abstract:** *Listeria monocytogenes* is a Gram positive rod-shape bacteria. Other foodborne pathogens, mostly, may cause typical symptoms of gastroenteritis. Unlike these microorganisms, *Listeria monocytogenes* infection may result in more serious symptoms, usually take in the form of meningitis or septicaemia. People with vulnerable immune system are in a bigger risk of being exposed. Furthermore, the bacterium is also severer than any other bacteria found in the food, with more resistant to heat, drying, or salty environment. This research was purposed to identify and characterize a sample named LM 25722248, which was isolated from fresh food factory in a city of United Kingdom. The isolate was characterized as a Gram positive bacterium, showed tumbling motility, and gave positive result for catalase test and haemolysin assay. Moreover, benefiting from molecular technology, it was possible to reveal that the isolate was *Listeria monocytogenes*.

**Keywords:** *Listeria monocytogenes*, foodborne illness, speciation PCR, characterization.

**Abstrak:** *Listeria monocytogenes* adalah bakteri Gram positif berbentuk batang. Patogen penyebab penyakit yang dibawa oleh makanan umumnya menimbulkan gejala khas gastroenteritis. Hal ini berbeda dengan *Listeria monocytogenes* yang dapat menyebabkan gejala yang lebih serius seperti meningitis atau septikemia. Dengan demikian orang yang memiliki sistem imun lemah akan lebih rentan terpapar cemaran bakteri tersebut. *Listeria monocytogenes* juga lebih sulit dihilangkan dibanding bakteri lain yang ditemukan dalam makanan, karena lebih resisten terhadap pemanasan, pengeringan, atau lingkungan yang mengandung garam. Penelitian ini bertujuan untuk melakukan identifikasi dan karakterisasi sampel bakteri yang diisolasi dari pabrik makanan segar yang berlokasi di salah satu kota di United Kingdom. Dengan metode biokimia, sampel yang disebut dengan LM 25722248 tersebut merupakan bakteri Gram positif, bersifat motil (*tumbling motility*), dan memberikan hasil positif terhadap uji katalase dan uji hemolisin. Lebih jauh lagi, dengan memanfaatkan teknologi molekuler maka dapat dinyatakan bahwa isolat LM 25722248 adalah *Listeria monocytogenes*.

**Kata kunci:** *Listeria monocytogenes*, penyakit bawaan makanan, PCR, karakterisasi.

## INTRODUCTION

FOOD safety is now a public responsibility as the food supply becomes increasingly globalized, resulting in greater availability and diversity of food. On the other side, new threats of food safety is developing. Antimicrobial resistance, new and

emerging pathogens, changes in environment, and changes in food production and distribution are all that challenge the food safety systems. World Health Organization estimated that there are 2 million people, mostly children, dead annually related to unsafe food<sup>(1)</sup>. In United Kingdom, it was estimated that around a million people suffer a foodborne illness; 20,000 people are hospitalized, and around 500 deaths caused by foodborne illness<sup>(2)</sup>. According

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to World Health Organization, foodborne illness are disease that infectious or toxic in nature and usually caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food or water<sup>(1)</sup>. In United Kingdom (UK), the priority pathogens to be identified are *Campylobacter*, which causes the largest number of cases each year, and *Listeria monocytogenes* (*L. monocytogenes*), which is responsible for the largest number of deaths<sup>(2)</sup>.

*L. monocytogenes* is a Gram positive bacteria that may cause listeriosis. It is dangerous as the outcome of the disease caused by the particular bacterium can be vary from mild to severe symptoms of nausea, vomiting, aches, fever, and, sometimes, diarrhea, or more deadly when it enters the bloodstream to the nervous system resulting in meningitis and other potentially fatal problems. People at risk for this disease are pregnant women, elderly, neonates and people with chronic illnesses as commonly they have more weak immune system than others<sup>(3)</sup>. Moreover, *L. monocytogenes* is a serious threat to the food industry because of its natures. This bacterium can survive and even grow at refrigerator temperature, tolerant of both low pH and high salt concentration. Hence, potential contamination sources may include food workers, incoming air, raw materials, and food processing environments. Among those, post-processing contamination at food-contact surfaces suggested to be the greatest threat to product contamination. Once contaminate the critical points, *Listeria* can settle and even form biofilm thus continuously generate contamination to the products. It is hardly surprising then that *L. monocytogenes* contamination turn out to be the leading reason of food recalls due to microbiological concerns lately<sup>(4,5)</sup>. This research was purposed to identify and characterize *L. monocytogenes* isolated from a product from fresh food factory.

## MATERIALS AND METHODS

**Bacterial Strains.** The bacterial strains used in this research are laboratory strain of *L. monocytogenes*, *Listeria innocua* (*L. innocua*), LM 00054 (*L. monocytogenes* isolate from Ireland) and LM 10403S (*L. monocytogenes* isolate from laboratory/type strain) which were supplied by Dr. Cath Rees (School of Biosciences, The University of Nottingham). Sample designated as LM 25722248 obtained from a fresh food factory. All *Listeria* species were grown at 37°C for 24-48 h (with shaking for broth culture) prior to performing the experiments.

**Growth media. Brain Heart Infusion (BHI, Oxoid, England) broth and agar.** BHI broth was

made by adding 37 g of the dehydrated medium into 1 L of RO water. This was mixed and then autoclaved at 121°C for 15 min to sterilise. BHI agar was made by adding 37 g of the dehydrated medium and 5 g of agar (Oxoid, England) into 1 L of RO water.

**Brilliance Listeria Agar (Oxoid, England) With Egg Yolk.** Brilliance Listeria Agar was made by adding 33.6 g of the dehydrated medium and 5% egg yolk to 480 mL of RO water. These two media were mixed and autoclaved at 121°C for 15 min to sterilise. The Brain Heart Infusion agar and Brilliance Listeria Agar were then poured into sterile petri dishes each, under aseptic conditions.

**Maximum Recovery Diluent (MRD, Oxoid, England).** MRD was made by adding 9.5 g of MRD powder to 1 L of RO water with a further step of sterilization using autoclave at 121°C for 15 min.

**Tris-HCl-EDTA (TE) Buffer.** The TE buffer was 1X TE buffer, consists of 10 mM Tris-HCl (pH 8) and 1 mM EDTA. The 10 mM of Tris-HCl was made by dissolving 1.211 g of Trizma base (Sigma-Aldrich, USA) to 1 L of RO water and adjusting its pH until 8 by adding dropwise of HCl in a stirring beaker while monitoring with a pH meter. As much as 0.372 g of EDTA (Fisher Scientific, UK) were then added into Tris-HCl solution to made 1X TE buffer. This was mixed and then autoclaved at 121°C for 15 min to sterilise.

**5X Lysis Buffer.** A 5X lysis buffer was made by mixing 12.5 mL of 250 mM of Tris-HCl, 25 mL of 250 mM EDTA, 2.5 g of lauroyl sarcosine (Sigma-Aldrich) (5%) and H<sub>2</sub>O up to 50 mL. A further step of sterilization used autoclave at 121°C for 15 min.

**Phosphate Buffered Saline (PBS, Oxoid, England) Solution.** PBS solution was made by adding 1 PBS tablet into 100 mL of RO water. The next process was sterilization using autoclave at 121°C for 15 min.

**Gram Staining.** Before performing staining, a thin layer of bacterial colony need to be prepared. A loop of MRD was placed on a clear labelled glass microscope slide. A very small sample of a bacterial colony was picked up stirred into the drop of MRD on the slide to create an emulsion. Heat fixing then carried out by allowing the smear to air dried and passing the slide through the flame of a bunsen burner two or three times. The smear was now ready to be stained. Slides were then placed in a rack and placed into crystal violet, as the primary stain, for 60 s. The slides were then washed with water prior to be placed in iodine for 30 s. The slides were then washed again with water before decolourized using methanol and decolourising was performed for 60 s. The slides were then placed in safranin, which acts as a counter stain,

for 30 s. The slides were then blotted dry and observed under a 100x oil immersion lens. Gram-positive cells are those that are stain purple and retained the crystal violet, whereas Gram-negative cells are pink resulted from the safranin<sup>(6)</sup>.

**Motility Test.** Bacterial motility was observed using microscope with a phase-contrast objectives and a condensor assembly to control illumination. A loopful of MRD was placed in a clean glass microscope slide. A small portion of bacterial colony was then sited in MRD and the emulsion was covered with a cover slip. Observation was carried out under 100x oil immersion lens. The bacterium and its structures appear darker than the background<sup>(7)</sup>.

**Catalase Test.** Catalase activity of bacteria was performed under Catalase Test Protocol from American Society for Microbiology<sup>(8)</sup>. The activity was detected using hydrogen peroxide. A loopful of bacteria was placed in a drop of hydrogen peroxide and the positive reaction appeared as vigorous bubbles which occurred within 10 s. *Escherichia coli* and LM 10403S were used as negative and positive controls, respectively.

**Haemolysin Assay.** Positive control in haemolysin assay was a mixture of 1450  $\mu$ L of H<sub>2</sub>O and 50  $\mu$ L of washed sheep blood. While, negative control (blank) was a mixture of 50  $\mu$ L of washed sheep blood, 1350  $\mu$ L of PBS and 100  $\mu$ L of supernatant of *L. innocua* culture. Next, a mixture of 50  $\mu$ L of washed sheep blood, 1350  $\mu$ L of PBS and 100  $\mu$ L of supernatant of LM 25722248 culture, together with each eppendorf of controls, were mixed gently by inversion and incubated at 37°C for 30 min. The solutions were then centrifuged at 13000 x g for 5 min at room temperature. The supernatant was transferred into a cuvette and the A<sub>540nm</sub> was measured. The A<sub>540nm</sub> value represents the intensity of the red coloured of the supernatant, which is proportional to the amount of lysed RBCs. The amount of RBCs and PBS used in the mixture solution should be adjusted to give the A<sub>540nm</sub> of positive control to be between 0.8 and 1.0<sup>(9)</sup>.

**DNA Extraction.** The extraction of genomic DNA was carried out using Wizard<sup>®</sup> Genomic DNA purification kit (Promega) and followed the provided

manufacturer instructions for isolation of genomic DNA from Gram positive and Gram negative bacteria.

**Speciation Polymerase Chain Reaction (PCR).** The protocol for speciation PCR was adapted from Somer and Kashi (2003). Speciation PCR mixture contained 1X flexi buffer (Promega), 2 mM MgCl<sub>2</sub> (Promega), 0.2 mM of each deoxynucleoside triphosphate (Promega), 1 U of GoTaq polymerase (Promega), 1  $\mu$ L of genomic DNA (50 ng/ $\mu$ L) and distilled water (HyClone HyPure molecular biology grade water, Thermo Scientific) up to the volume of 25  $\mu$ L. The primers used were 1 l of 10 pmol/ $\mu$ L concentration of each LIS-F, MONO5-F, MONO7-Fa, and 1.7 l of 10 pmol/ $\mu$ L of LIS-R (Eurofins MWG Operon, Ebersberg, Germany). The PCR reactions performed with a PCR thermal cycler (Techne TC-312, UK) under following conditions: 1 cycle at 95°C for 5 min; 5 cycles at 95°C for 45 s, 61°C for 45 s, and 72°C for 45 s; 20 cycles at 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s; and 1 cycle at 72°C for 7 min. The PCR products were separated on 1% (w/v) agarose (Fisher BioReagents, USA) gel, in 50-70 V and scored relative to 100 bp DNA size ladder (Promega, Madison, USA). Table 1 shows the primers used.

## RESULTS AND DISCUSSIONS

Reduction of foodborne illness or foodborne disease is a key objective to ensure the food safety. Therefore, it is necessary to conduct a research to track the contamination possibility of *L. monocytogenes* within the industrial flow process. In this research, LM 25722248, a sample isolated from product (pepper) in a fresh food factory, had been identified and characterized.

**Isolate Growth in Selective Media and Gram Staining.** According to Food and Drug Administration (FDA, USA), first detection of *L. monocytogenes* in food is examining the sample on selective media for Listeria<sup>(10)</sup>. In this research, the selective media used was Brilliance Listeria Agar which enables identification of Listeria species based on their utilisation of a chromogenic substrate. Brilliance Listeria Agar contains chromogen X-glucoside

Table 1. Primer sequences used in speciation PCR<sup>(12)</sup>.

| Primers  | Primer Sequence (5'→3') | Descriptions  |
|----------|-------------------------|---|
| LIS-F    | AGCTTGCTCTTCCAAAGT      | <i>L. monocytogenes</i> , <i>L. innocua</i> , <i>L. seeligeri</i> ,<br><i>L. welshimeri</i> |
| MONO5-F  | GCTAATACCGAATGATAAGA    | <i>L. monocytogenes</i> sequence variant B<br>(serotype 4a)                                 |
| MONO7-Fa | GGCTAATACCGAATGATgAA    | <i>L. monocytogenes</i> sequence variant A (all<br>other serotype)                          |
| LIS-R    | AAGCAGTTACTCTTATCCT     | All Listeria spp.   |

which is cleaved by *Listeria* species as they have  $\beta$ -glucosidase. The cleavage could be seen as a blue/green colonies for *L. monocytogenes* (see Figure 1, the a arrow). Further detection came out from a white halo (a clear zone) around the colony (see Figure 1, the b arrow). The medium contained the egg yolk, therefore white halo occurred as lecithin in the egg yolk was hydrolyzed. It was useful as an indicator for pathogenicity since the presence of the enzymes that hydrolyse lecithin are associated with virulence in *Listeria* species<sup>(11)</sup>. Then, LM 25722248 was characterized using Gram staining method. Figure 2 showed that the bacterium was a Gram positive and had a rod shape.

**Motility and Catalase Tests.** The next characterizations were motility and catalase tests. LM 25722248 revealed positive for both tests. The bacterium showed tumbling motility under wet mount observation. On addition of hydrogen peroxide for catalase test, LM 25722248 colony formed

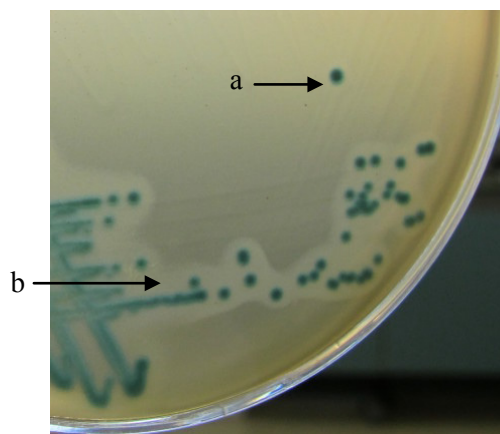


Figure 1. *L. monocytogenes* colonies on Brilliance Listeria Agar. The "a" arrow shows the blue/green colony while the "b" arrow shows the white halo.

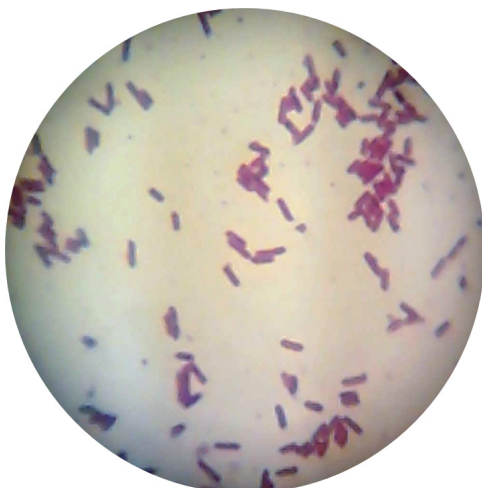


Figure 2. Gram staining of LM 25722248.

effervescence, which was observed also on LM 00054 and LM 10403S, but was not detected on *Escherichia coli* colony (Figure 3). LM 25722248 was catalase positive which gave similar result as positive control (LM 00054 and LM 10403S).

**Haemolysin Assay.** The haemolysin assay used a sheep blood that needed to be washed first in order to produce a stable RBCs resuspended in PBS, and remove all lysed cell contents containing haemoglobin. Hence, any lysis observed was resulted from the haemolytic activity of the bacterium rather than due to spontaneous lysis of friable RBCs. Other condition that might lysed the cell was the low pH of the culture media resulted from overnight growth of *L. monocytogenes*. Therefore, *L. innocua* was used as a negative control strain to determine the basal level of lysis, sample with absorbance higher than this negative control would be considered as a haemolysin positive. On the contrary, 100% haemolysis was expressed by the positive control which contained RBCs and water only. The  $A_{540nm}$  reading measurements of LM 25722248 isolate, compared to other two isolates (LM 00054 and LM 10403S), were presented in Table 2. LM 25722248 and LM 10403S isolates were haemolysin positive as they exhibited a high absorbance compared to the negative control, which meant a high red coloured intensity resulted from lysed RBCs. The LM 00054 isolate was not haemolysin positive as it had no difference of absorbance with the negative control.

**Speciation PCR.** Further characterization of the LM 25722248 isolate was performed using PCR for more rapid, specific and sensitive detection of *L. monocytogenes* in food products. The PCR was based on DNA sequences and primer pairs that were found within the 16S subunit of the rRNA gene and are

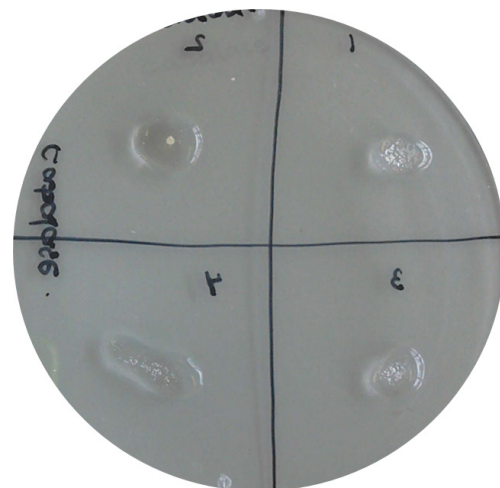
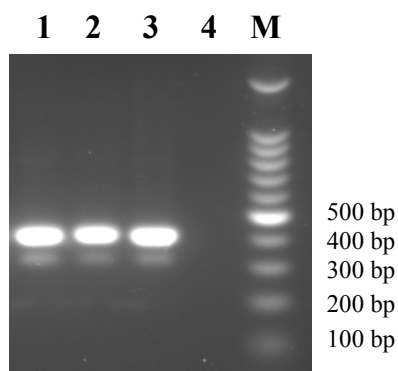


Figure 3. Isolate colonies that performed catalase test were as follows: 1). *Escherichia coli*, 2). LM 00054, 3). LM 10403S, 4). LM 25722248.

**Table 2. The  $A_{540nm}$  reading measurements of haemolysis assay of sample (LM 25722248), *L. monocytogenes* isolates (LM 10403S and LM 00054) and *L. innocua* as negative control.**

| Isolate           | Absorbance ( $A_{540nm}$ ) measurements |       |       |       | Haemolysin |
|-------------------|---|-------|-------|-------|------------|
|                   | $A_1$                                   | $A_2$ | $A_3$ | Means |            |
| LM 25722248       | 0.17                                    | 0.19  | 0.22  | 0.19  | +          |
| LM 10403S         | 0.15                                    | 0.17  | 0.14  | 0.15  | +          |
| LM 00054          | 0.01                                    | 0.01  | 0.01  | 0.01  | -          |
| <i>L. innocua</i> |   |       |       | 0.00  | -          |



**Figure 4. A 1% agarose gel electrophoresis of DNA fragments generated by speciation PCR. Lane 1: LM 10403S, lane 2: LM 00054, lane 3: LM 25722248, lane 4: negative control, lane M: 100 bp ladder.**

specific to *Listeria* genus and to *L. monocytogenes*. Firstly, the DNA of LM 25722248 was extracted prior to perform PCR reaction. Electrophoresis of PCR products in 1% agarose gel resulted as shown in Figure 4. The expected band products were 400 bp for *Listeria* genus and 287 bp for *L. monocytogenes*<sup>(12)</sup>. It could be seen from the result that the three isolates in lane 1, lane 2 and lane 3 possessed the 400 bp band and one band in 300-400 bp size, but lack of 287 bp band. The electrophoresis gave an unexpected result. It was probably because the absence of IVA-F and MG-F primers (5'-AGCTTGCTCTTCCAATCT-3' and 5'-GCTTGCTCCTTTGGTGC-3', respectively), which were used on the referred journal. Recall that lane 1 (LM 10403S) and lane 2 (LM 00054) were *L. monocytogenes*, thus lane 3 (LM 25722248) with similar bp was *L. monocytogenes* as well.

## CONCLUSION

LM 25722248 was a sample isolated from product of fresh food factory. Identification and characterization of isolate suggested that it was *L. monocytogenes*, a Gram positive bacterium, showed tumbling motility, and gave positive result for catalase test and haemolysin assay. Furthermore, benefiting from molecular technology by speciation PCR it was possible to reveal that the isolate was *L. monocytogenes*.

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