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The investigation of molecular characterization of presumptive *Listeria monocytogenes* isolates from a food-processing environment

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Summary

Background: *Listeria* is a Gram-positive, non-spore forming, facultative anaerobic intracellular bacterium. The most important pathogens in mammals include *Listeria monocytogenes* and *Listeria ivanovii*. The former generally causes disease and death in both humans and animals while the latter performs sporadically and primarily causes illness in ruminants. **Aims:** The aim of this project was to use conventional and molecular techniques to determine whether the provided samples were *L. monocytogenes*, and whether they were genetically similar or different. **Methods:** The provided presumptive *Listeria* cultures isolated from industrial processed food are conventionally assumed to be *L. monocytogenes*. All samples were cultured on brain heart infusion agar and broth first and then on blood agar. Later, *hly* gene amplification was applied. **Results:** The provided culture phenotypically resembled *L. monocytogenes* as it caused haemolysis on blood agar plates; however, the absence of the *hly* gene revealed that they were genotypically different. *16S rRNA* confirmed three species of *Listeria* species including *L. grayi*, *L. welshimeri* and *L. ivanovii*. The results from *16S rRNA* sequencing confirmed the results obtained from *hly* gene amplification. **Conclusion:** Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC PCR) confirmed that all bacterial cultures were isolated from different sources depending on their ERIC PCR profile variation.

Key words: Isolation, *Listeria monocytogenes*, Molecular characterisation

Introduction

Listeria is a Gram-positive, non-spore forming, facultative anaerobic intracellular bacterium (Orsi *et al*., 2011). It is 1-1.5 μm long and 0.5 μm wide (Marian *et al*., 2012). The genus *Listeria* has encompassed a number of species: *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. ivanovii*, *L. monocytogenes*, and the recently identified species, *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis* and *L. fleischmannii* (Hellberg *et al*., 2013). The known mammalian pathogenic species within the *Listeria* genus is *L. monocytogenes*, which generally causes disease and death in both humans and animals (Jadhav *et al*., 2012), and *L. ivanovii*, which performs sporadically and primarily causes illness to ruminants (Orsi *et al*., 2011). Based on ribotypes and virulence gene *hly*, *L. monocytogenes* are classified into four genetic lineages (Chen *et al*., 2011). They comprise of two main lineages I and II to which almost all serotypes isolated from foods and patients belong. III and IV lineages are infrequent and are mainly isolated from non-primate mammals and ruminants (Chen *et al*., 2013). There are a number of serotypes that belong to each lineage including 1/2b, 1/2a, 1/2c, 3a, 3b, 3c and 4b associated with lineage I and II, while 4a, 4b, and 4c belong to lineage III and IV (Orsi *et al*., 2011). *Listeria monocytogenes* is widely present in the environment, water and a wide range of food processing environments and can supposedly appear on all raw food products (Lambertz *et al*., 2013). It has the capability to acclimatize and persist in a wide range of different extreme environments (high salt concentration 10% NaCl, pH range 4.5-9.6 and temperature range -1 to 45°C) (Marian *et al*., 2012). Nevertheless, pasteurization kills all *Listeria*; hence the contamination of dairy processing plants is usually a consequence of post pasteurization processes (Jadhav *et al*., 2012).

The disease caused by *L. monocytogenes* is known as listeriosis and can have two forms. The invasive form causes illness primarily among at-risk populations such as pregnant women, infants, elderly and immune-suppressed persons (Laksanalamai *et al*., 2012). The other form, febrile gastroenteritis, is a non-invasive variant and a self-limiting form of listeriosis, which affects healthy people and represents itself by aches, self-limiting fever, diarrhoea, nausea and fatigue (Laksanalamai *et al*., 2012).

Since the first foodborne listeriosis outbreak in 1981 (Schlech III *et al*., 1983), cases of listeriosis have increased in humans (Acciari *et al*., 2011). The Centres for Disease Control and Prevention stated that around 1600 invasive cases of listeriosis with nearly 255 deaths occur annually in the United States (Laksanalamai *et al*., 2012). Whereas in the in European Union, 2-10 cases are reported per million people annually with a large fatality ratio which can be more than 30% (Mammina *et al*., 2009). In Australia 3 cases are expected per 100,000 people with high fatality rate of 20-30% (Hogg *et al*.,

2013).

The diagnosis of *L. monocytogenes* is challenging because it is related to other species and does not have specific symptoms. *Listeria* species are not distinguished by any regulatory body such as the US Department of Agriculture (USDA), Food Standards Australia and New Zealand and Food and Drug Administration (FDA), because it is suggested that the non-pathogenic species may be involved in the pathogenicity process of listeriosis. Therefore, sensitive, reproducible and robust methods are needed to detect *L. monocytogenes* in food industries (Jadhav *et al*., 2012).

To detect *L. monocytogenes*, several methods are available including culture based techniques and immune-based techniques. The most reliable and sensitive methods that rely on genetic rather than phenotypical information are molecular based techniques of detection such as DNA microarrays, multiplex polymerase chain reaction (PCR), real time PCR based methods and conventional PCR (Jadhav *et al*., 2012).

The aim of this project was to determine whether the provided samples were *L. monocytogenes* or not, and whether they were genetically similar or different using both conventional and molecular techniques.

Materials and Methods

The provided samples were 11 presumptive *Listeria* cultures isolated from a food-processing environment. The appearance of these isolates on selective media morphologically indicated that the samples were presumptively *L. monocytogenes*. Samples were designated MC1, MC3, MC4, MC5, MC6, MC7B, MC8, MC9A, MC9B, MC10, and MC12.

Bacterial cultures on agar and in broth

Each sample was streaked on brain heart infusion (BHI) agar (Oxoid Basingstoke, United Kingdom) plates and incubated for 24 h at 37°C for further subculturing. The next day, BHI broth media was prepared by inoculating the bacterial sample from previously cultured bacteria and incubated for 24 h at 37°C to prepare for DNA extraction. In addition, ready-to-use horse blood agar plates were provided, on which all bacterial samples were subcultured. Each sample was streaked on the blood agar plates and incubated at 37°C for 24 h to show the haemolysis ability of bacteria. Gram stain techniques were also applied according to Prophet *et al*. (1992).

DNA extraction

DNA extraction was performed using Wizard Genomic DNA Purification Kit (Promega Company, USA).

Polymerase chain reaction

PCR for hly gene

PCR was used for the amplification of the *hly* gene in order to determine the virulence gene of *L. monocytogenes*. Primers and conditions used in the *hly* PCR were described by Zhang *et al*., (2009). Table 1 lists the primers used in the *hly* amplification. These primers produced PCR products around 596 bp. Each 25 µL of reaction contained 12.5 µL of Mega mix (Promega, USA), 0.5 µL of each primer, 10.5 µL nuclease-free water and 1 µL of bacterial genomic DNA. The condition for *hly* PCR is detailed in Table 2. The product was analysed using 1% agarose gel electrophoresis.

PCR for 16S rRNA gene

PCR for *16S rRNA* gene was achieved to detect different types of *Listeria* by sequencing the PCR product. The two universal primers are shown in Table 1. PCR reaction was performed in 25 µL PCR reaction mixture containing 1 µL of bacterial genomic DNA, 0.5 µL of each primer, 12.5 µL of Mega mix (Promega, USA), and 10.5 µL nuclease-free water. PCR conditions were described previously by Rohwer *et al*. (2001).

Enterobacterial repetitive intergenic consensus (ERIC) PCR

ERIC PCR was achieved to generate DNA fingerprints of all samples by applying the technique described by Duan *et al*. (2009). The two primers used in the PCR are detailed in Table 1.

Table 1: List of primers used in this project

Primers	Sequence (5´-3´)	Product size (bp)	References
hly1F	AGCACAACAAACTGAAGCAAAGGA	596	Zhang *et al*. (2009)
hly2R	ATTGTGATTCACTGTAAGCCATTTCGTCAT		
16s RNA 27F	AGAGTTTGATCMTGGCTCAG	1600	Rohwer *et al*. (2001)
16s RNA 1492R	TACGGYTACCTTGTTACGACTT		
ERIC1	ATGTAAGCTCCTGGGGATTCAC		Duan *et al*. (2009)
ERIC2	AAGTAAGTGACTGGGGTGAGCG		

Table 2: *hly* PCR condition

Step	Number of cycles	Temperature (°C)	Time
Initial denaturation	1	94	5.0 min
Denaturation	35	94	30 s
Annealing		58	30 s
Extension		72	30 s
Final extension	1	72	7 min

Table 3: ERIC PCR condition

Step	Number of cycles	Temperature (°C)	Time (min)
Initial denaturation	1	94	5.0
Denaturation	35	94	1
Annealing		54	1
Extension		72	8
Final extension	1	72	8

PCR reaction was performed in 25 µL reaction mixture containing 1 µL (30 ng/µL) of bacterial genomic DNA, 1 µL (25 pmol/µL) of each primer and 12.5 µL Mega mix (Promega, USA) and 9.5 µL nuclease-free water. PCR was performed in thermocycler (Bio-Rad) applying the conditions detailed in Table 3. The PCR product was analysed by agarose gel electrophoresis.

Preparation of DNA for sequencing

Cleaning up DNA from agarose gel

After the amplification of *16S rRNA* gene was completed, the DNA was extracted from the gel using the "Wizard SV Gel and PCR clean up system" (Promega, USA). Afterwards, DNA concentration was performed by a Biowave2 spectrophotometer. Finally, following the Australian Genome Research Facility (AGRF) sequencing centre, the total volume of the reaction was 12 µL containing the primer, aliquot DNA and sterile distilled water. The recommended DNA concentration to sequence the product more than 800 bp was 30-75 ng/µL. The appropriate volume of each sample was added to the solution, 1 µL of 27 F primer and completed to 12 µL with sterile distilled water.

Alignment of DNA sequences

The sequences were pasted into the text area of the page to find the closest match with DNA sequences in the Gen Bank database "BLAST".

Results

Culturing of bacteria on blood agar and Gram stain

All bacterial samples showed β-haemolysis after incubation for 24 h at 37°C (Fig. 1). They appeared as Gram-positive, short, rod shaped bacterium under the microscope. The Gram stain of bacterial samples is important because presumptive *Listeria* is believed to be Gram-positive (Atil *et al*., 2011).

hly gene PCR

To confirm the type of *Listeria* genotypically, DNA was extracted from the presumptive *Listeria* samples and analysed for the presence of the *hly* gene by amplifying this gene. The PCR products for all samples were run on 1% agarose gel as shown in Fig. 2. All bacterial samples in wells 3 to 13 were negative for the *hly* gene. whereas a clear DNA band, around 560 bp in size, appeared in well 15 as control DNA for *L. Monocytogenes*.

Due to the importance of the *hly* gene for the identification of *L. monocytogenes* (Liu, 2008), *hly* PCR was applied to all samples. The results of this experiment indicated that none of the provided samples were *L. monocytogenes*, due to the fact that the *hly* gene does not exist in all bacterial samples and the agarose gel was clear from any bands except for control DNA band in *L. monocytogenes*.

PCR for *16S rRNA* gene and sequencing

To verify the results obtained from the *hly* PCR, amplification of the *16S rRNA* gene was carried out for the bacterial samples and the PCR products were sequenced. After running the PCR products on 1% agarose (Fig. 3), a clear band appeared for all samples around 1600 bp on Lines 2 to 12. The DNA bands of *16S rRNA* for all samples were cleaned up from the agarose gel after amplification and prepared for sequencing. The DNA sequencing was performed at the AGRF sequencing centre (AGRF, Melbourne, Australia). The size of *16S rRNA* sequences obtained was around 1 kb.

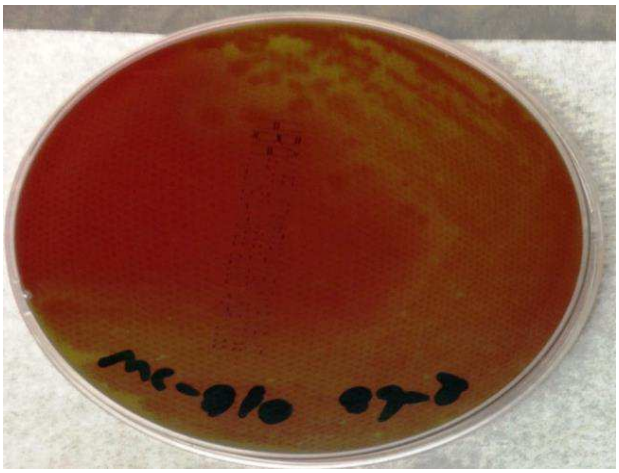

Fig. 1: *Listeria* culture on blood agar showing β-haemolysis

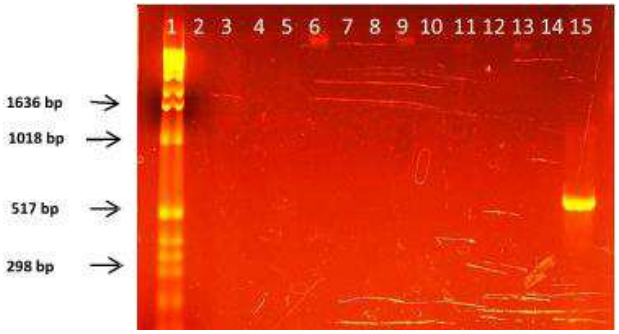

Fig. 2: hly PCR products on 1% agarose gel. Line 1 (1 kb), Lines 3-13 (samples MC1, MC3, MC4, MC5, MC6, MC7B, MC8, MC9A, MC9B, MC10, and MC12), Line 15 (DNA of *L. monocytogenes* as control), and Lines 2 and 14 (empty)

Table 4: *Listeria* species obtained from the BLAST alignment of *16S rRNA* gene sequences

Samples	Alignment bacteria	Strain	Identical (%)	Accession No.
MC7B	*Listeria grayi*	ATCC 25401	99	JN852815.1
MC9B	*Listeria welshimeri*	SLCC5334	99	AM263198.1
MC10	*Listeria ivanovii*	PAM 55	99	FR687253.1

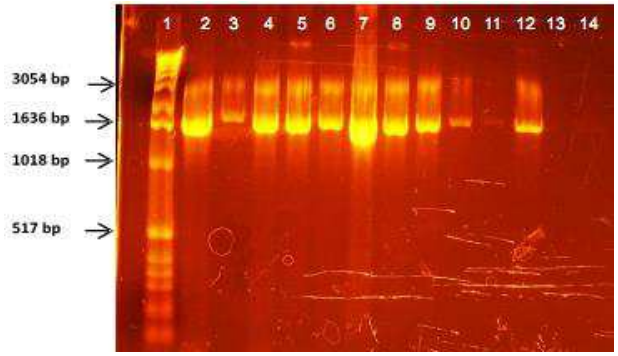

Fig. 3: PCR of *16S rRNA* on 1% agarose gel. Line 1 (1 kb), Lines 2-12 (samples MC1, MC3, MC4, MC5, MC6, MC7B, MC8, MC9A, MC9B, MC10, and MC12)

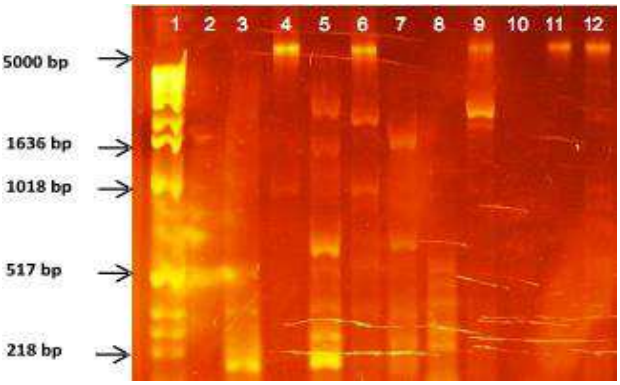

Fig. 4: ERIC PCR on 1% agarose gel. Line 1 (1 kb), and Lines 2-12 (samples MC1, MC3, MC4, MC5, MC6, MC7B, MC8, MC9B, MC9A, MC10, and MC12)

Basic Local Alignment Search Tool (BLAST) search tool was used to determine the closest matching bacteria to samples' *16S rRNA* sequence. Only three samples identified with the species' level (Table 4). The remaining samples shared a similarity between 77% and 80% with *L. grayi*.

ERIC PCR

ERIC PCR of genomic DNA from presumptive *Listeria* samples isolated from processing food environments produced fingerprint profiles comprising of two to seven bands of sizes ranging 140-5500 bases as shown in Fig. 4.

Discussion

Culturing bacteria on blood agar and Gram stain

Previous studies suggest that only three species of *Listeria, L. monocytogenes*, *L. ivanovii*, and *L. seeligeri,* typically cause β-haemolysis on blood agar (Allerberger, 2003). They cause the lysis of red blood cells of most mammals and the haemolysis is most frequently revealed using blood agar plates containing horse or sheep blood. β-haemolysis occurs as a result of *Listeriolysin* O (LLO, ILO and SLO) protein encoded by the *hly* gene, which is located within the cluster of virulence genes, hence revealing their activity (Liu, 2008). The important indication of test is isolate the virulent types of *Listeria* phenotypically from non-pathogenic types because the haemolytic reaction is only performed by the virulent species. Therefore, it is a significant step towards the differentiation of *L. monocytogenes* and *L. ivanovii* from other types of *Listeria* (Liu, 2008).

Evaluating β-haemolysis can be challenging in *L. monocytogenes* because the formed clearing zone is very small and in some strains, the colonies need to be removed in order for the haemolysis to be identified. In addition, haemolysis is seen in unusual cases in the non-pathogenic type of *Listeria* (Liu, 2008). Moreover, contamination with other haemolytic bacteria such as *Streptococcus pyogenes* while preparing the culture media or streaking bacteria on agar, may yield false positive results (Liu, 2008).

It is important to know the Gram stain of bacterial samples because the provided presumptive *Listeria* are supposedly Gram-positive (Atil *et al*., 2011).

hly gene PCR

Due to the importance of the *hly* gene for identifying *L. monocytogenes* (Liu, 2008), *hly* PCR was applied to all samples. The results of this experiment indicated that none of provided samples were *L. monocytogenes* because the *hly* gene does not exist in all bacterial samples and the agarose gel was clear from any bands except for the control DNA band of *L. monocytogenes*.

PCR for *16S rRNA* gene and sequencing

16S rRNA is a small-subunit rRNA molecule in prokaryotic cells with a sedimentation coefficient of 16S, and it is the most conserved gene around 1600 bp in all cells. It is most frequently used to characterize bacteria (Hellberg *et al*., 2013). For phylogenetic purposes of higher taxonomic orders such as phylum, family, and genus, the *16S rRNA*'s preserved regions are used as targets for most bacteria, whereas the variable sequence regions are significant for isolating the genus (Liu, 2008). *16S rRNA* gene sequencing is a particularly irreplaceable method when recognizing poorly defined or phenotypically unusual strains (Liu, 2008). All samples produced clear bands at around 1600 bp, which is an entire *16S rRNA* gene (Hellberg *et al*., 2013).

When alignment is performed to detect the closest matched bacteria, identifying the percentage is considered to be a significant parameter. The similarity

between aligned sequences should be ≥99% to confirm the identity of bacterial species, ≥95 to <99% to recognise the genus of bacteria, and less than 95% to identify the bacteria at family level (Bosshard *et al*., 2003).

ERIC PCR

ERIC sequences are extremely conserved as an intergenic functional repeat region of DNA with 124-147 bp size. They are present in a varied range of bacterial genomes comprising *Listeria* that signify prospective sites to bind primers for PCR amplification in order to identify bacterial species and strain (Soni *et al*., 2013). Different profiles imply diverse sources for bacteria isolation, which in turn means that the bacteria are isolated from different sources (Liu, 2008).

Samples MC7B and MC9B yielded two different profiles (as shown in Fig. 4, Line 7 and 9), supporting the idea that the sequence data represent two different species of *Listeria*. The diverse ERIC profile of other samples indicates that they may form different species of *Listeria*.

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