Using RAPD-PCR as molecular assessment on the performance of CHROMAgar™ Listeria and PALCAM agar on isolation of Listeria spp. and L. monocytogenes from foods

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Abstract: In this study, Listeria spp. were isolated from naturally contaminated samples of beef burger, minced beef and sliced cheese using PALCAM and CHROMagar™ Listeria before confirmation using PCR on hlyA and LLO toxin genes specific to L. monocytogenes. Identification of isolates showed a total of 45 isolates of Listeria spp. and two L. monocytogenes. All the 47 isolates were then subjected to RAPD analysis using two oligomers (OPA14 and OPA15) and fingerprint clustering was able to cluster the L. monocytogenes from Listeria spp. based on isolation from agar types as well as L. monocytogenes from Listeria spp. Studies showed that OPA14 and OPA15 are useful for rapid discrimination of Listeria spp. and L. monocytogenes. The differences observed on the isolation of Listeria spp. from PALCAM and CHROMagar™ Listeria that may have an impact on epidemiological studies.

Keywords: L. monocytogenes, RAPD fingerprinting, PALCAM agar, CHROMagar™ Listeria

Introduction

Listeria monocytogenes is a major concern in food safety because it can cause human listeriosis with mortality rate of up to 25% in susceptible individuals (Farber and Peterkin, 1991). L. monocytogenes is Gram positive, rod-shaped, with 13 serovar designations (Seeliger, 1958; Donker-Voet, 1972) which are 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Although the 13 serovars have been associated with outbreaks, serovar 1/2a, 1/2b and 4b are the causative agents for most human listeriosis (Farber and Peterkin, 1991). In L. monocytogenes, three lineages have been established which are lineage I (serotypes 1/2b, 3b, 4b, 4d and 4e), lineage II (1/2a, 1/2c, 3a and 3c) and lineage III (4a and 4c) (Nadon et al., 2001) which indicate the diversity of strain as well as the potential competitive fitness among strains.

L. monocytogenes is ubiquitous in environment and it is found in silage, sewage and farms (Farber and Peterkin, 1991; Hoff, 2003) but human listeriosis has been associated with food safety because of contamination in various types of foods especially ready-to-eats (Sahilah et al., 2010; Yousr et al., 2010). This is because such products are not subjected to thermal treatments and can be kept refrigerated that allow opportunities for the pathogen to grow to a dangerous level.

The increasing frequency of literature on evaluation of the existing protocols and methods for isolation and detection of L. monocytogenes (Busch and Donnelly, 1992; Ryser et al., 1996; Bruhn et al., 2005) underline the importance of detection and isolation method for this bacterium in foods regardless for food safety or epidemiological studies purposes. Identification and isolation of L. monocytogenes have been traditionally isolated on various agars such as PALCAM (polymyxin-acriflavine-lithium-chloride-ceftazidime-aesculin-mannitol) agar, modified Oxford agar, ALOA™, CHROMagar™ Listeria, blood agar and etc. Identification of isolates on agar is dependent on the substrate utilization such as cleavage of PI-PLC of L-α-phosphatidyl-inositol to form a white precipitation zone around colony in combination of chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside applied in CHROMagar™ Listeria, and cleavage of aesculin and breakdown of the product, aesculin with ferric ion to form grayish-greenish colonies with brown-black halo center in PALCAM agar. The differences in the substrate utilization and selectivity of the agar can also play
a role in differentiating the genotypic characteristics and can be traced via molecular methods.

Randomly amplified polymorphic DNA (RAPD) is a rapid and highly promising tool for discrimination of L. monocytogenes strains (Boerlin et al., 1995). Application of RAPD in epidemiology studies have been widely used (Giovannacci et al., 1999) and extended into other studies for validation or comparison of methods for the reproducibility of published primers for L. monocytogenes detection (Aznar and Alarcon, 2002). Other than that, investigations on the association of isolates in foods and clinical incidences showed the diversity of L. monocytogenes was unable to cluster the causative agent to the source (Martinez et al., 2003; Aurora et al., 2009). The epidemiological tracing may be affected by enrichment media or agar as found in some studies. For example, studies by Gracieux et al. (2003), showed that selective agars such as PALCAM, Oxford, Rapid L. mono(RLM) and ALOA Listeria has higher count of virulent L. monocytogenes compared to the nonvirulent strains while Bruhn et al. (2005) showed the bias in the University of Vermont broth enrichment in Lineage 2 L. monocytogenes. Due to the potential bias that may exist in enrichment broth, this study extends the knowledge to investigate the potential bias on PALCAM and CHROMagar™ Listeria. To the best of our knowledge, the exact profile on the preferences of Listeria spp. isolation based on these two agars have not been carried out and this represent the first study on differentiating the Listeria spp. and L. monocytogenes isolated on PALCAM and CHROMagar from the same sample via molecular fingerprinting. Therefore, the objective of the study is to conduct the assessment on the preference of PALCAM and CHROMagar™ Listeria in isolation of Listeria spp. in food samples using RAPD fingerprinting.

Materials and Methods

Sampling and enrichment

Total of twenty samples (4 cheeses, 8 beef burgers and 8 minced beefs) were purchased randomly from supermarkets for isolation and detection of L. monocytogenes. Sample processing was carried out based on FDA-BAM method. Briefly, 10 g of sample was homogenized with Buffered Listeria Enrichment broth (BLEB) for 4 h at 30°C followed by the addition of selective supplements (50 mg/L cycloheximide, 15 mg/L acriflavine HCl, 40 mg nalidixic acid) and further incubated at 30°C up to 48 h. Each sample was then streaked on PALCAM and CHROMagar™ Listeria before incubated at 30°C and 37°C for 48 and 24 h respectively. Single isolates were selected from the plates and further streaked on similar agar to ensure pure single colony.

DNA extraction, PCR, RAPD and gel electrophoresis

Single colonies obtained from the agar were streaked on TSAYeast (0.6% yeast) and incubated overnight in 37°C for 12 – 16 h. Colonies grown on the TSA were collected in sterile 1.5 mL microcentrifuge tube with 1 mL of sterile distilled water. Tubes were centrifuged at 12,000 rpm for 3 min with a washing step. Pellets were collected and resuspended with 400 μL of sterile distilled water and homogenized with vortex until no clumps were observed. The tubes were transferred into a dry bath at 100°C for 15 min and quickly transferred into -20°C for 15 min for crude cell lysis method for DNA extraction (Lee et al., 2009; Ponniah et al., 2010). Tubes were then subjected to centrifugation at 10,000 rpm for 5 min and supernatant was transferred into new sterile tubes and kept at 4°C to be used while preparation of PCR cocktail was carried out.

Identification of Listeria spp. and L. monocytogenes was carried out using primers: L1-U1 (Listeria spp.), LM1-LM2 (L. monocytogenes) as multiplex identification and multiplex toxin genes detection in L. monocytogenes (IAP1-IAP2) and (LMA-LMB) primers. PCR profiles and cocktails were prepared as shown in Table 1. Twenty five oligomers were screened for RAPD and the primers with most bands were selected to be used for RAPD fingerprinting. OPA 14 and OPA 15 provided best band on L. monocytogenes ATCC 19112 (serovar 1/2c from human) control culture therefore these two primers were used for RAPD fingerprinting of the isolates.

Ten μL of RAPD-PCR products was pipetted into the agarose gel (1.0%) using 0.5× TBE buffer and subjected to gel electrophoresis for 1 h 52 min at 80 V and 1 kb ladder (PROMEGA, USA) was used as the DNA marker reference. Gel viewing was carried out using GeneSnap (SYNgene, USA) and gel pictures were saved as TIFF file for further analysis on GelCompar II (Applied Maths, Belgium).

Analysis of RAPD fingerprinting

Gel image was carried out based on manual by GelCompar II (Applied Maths, Belgium). The dendogram was constructed using Pearson’s correlation and UPGMA on similarities of bands. Individual dendogram was constructed based on OPA 14 and OPA 15 and a combination of two oligomers for composite fingerprint profiling.

Calculation of discriminatory index (D) was
Results and Discussion

In present study, a total of 20 samples were appraised for detection of *L. monocytogenes* using microbiological plating method. With regards to the small number of sample, it should be noted that the emphasis of study was placed on the differences in isolation preference based on the two selective agents (nalidixic acid to suppress gram-negative bacteria and acriflavin for suppression of other gram positive bacteria) in PALCAM that reduces the competing flora in the food matrix while CHROMagar is known to be highly sensitive to differentiate *L. monocytogenes* from *L. innocua* with formation of white halo around the colony. The description of the identified 47 isolates were summarized in Table 2 to indicate reference key, source, type of samples, identification and agar used for isolation.

Three dendrograms were generated from the RAPD fingerprinting; OPA14 (Figure 1) and OPA15 (Figure 2) individually and combination of OPA14 and OPA15 (Figure 3). The discriminatory index for OPA14 was 0.492 and OPA15 at 0.930. The dendogram generated showed similarity of 30% to 100% for combination of primers, 40% to 98% for OPA14 was 0.492 and OPA15 at 0.930. The discriminatory index based on the following formula according to Hunter and Gaston (1998):

\[ D = 1 - \frac{1}{N(N - 1)} \sum_{j=1}^{s} n_j (n_j - 1) \]

where D is the discrimination index, N is the total number of strains in sample population, s is total number of types described, and \( n_j \) is the number of strain belonging to the \( j \)th type.

### Table 1. Details of PCR primers, profiles and sequences used for identification and RAPD fingerprinting analysis of *Listeria* spp. and *L. monocytogenes*

<table>
<thead>
<tr>
<th>Target</th>
<th>For</th>
<th>Sequence 5′-3′</th>
<th>Product size (bp)</th>
<th>PCR cocktail</th>
<th>PCR profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16sRNA</td>
<td>Identification of <em>Listeria</em> spp.</td>
<td>U1 – CAG CMG CCG CGG TAA TW C</td>
<td>916</td>
<td>1× GoTaq™ Green buffer</td>
<td>94°C – 5’</td>
<td>Border et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Identification of <em>L. monocytogenes</em></td>
<td>LMI-1 CCT AAG ACC CCA ATC CTC</td>
<td>702</td>
<td>1.5 mM MgCl₂ buffer</td>
<td>94°C – 30 cycles</td>
<td>Border et al., 1990</td>
</tr>
<tr>
<td>hly A</td>
<td>Identification of <em>L. monocytogenes</em></td>
<td>IAP1 - ACA AGC TGC ACC TGT TGC AG</td>
<td>131</td>
<td>1.5 mM MgCl₂ buffer</td>
<td>94°C – 2’ 30 cycles</td>
<td>Kohler et al., 1990</td>
</tr>
<tr>
<td>Invasion-associated protein</td>
<td>Identification of <em>L. monocytogenes</em></td>
<td>IAP1 - ACA AGC TGC ACC TGT TGC AG</td>
<td>131</td>
<td>1.5 mM MgCl₂ buffer</td>
<td>94°C – 2’ 30 cycles</td>
<td>Kohler et al., 1990</td>
</tr>
<tr>
<td>Listeriolysin O (–α haemolysis, listeriolysin O)</td>
<td>Identification of <em>L. monocytogenes</em></td>
<td>LMI – CCG AGG TTC CGC CAA AAA TG LMB – CCT CCA CAG TGA TCG ATG TT</td>
<td>234</td>
<td>0.5 Unit Taq Polymerase</td>
<td>94°C – 1’ 30 s</td>
<td>Furrer et al., 1991</td>
</tr>
<tr>
<td>DNA RAPD</td>
<td>OPA14 - TGT GTG CTG G</td>
<td>4 μL DNA</td>
<td>1× GoTaq™ Green buffer</td>
<td>94°C – 4’</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>DNA RAPD</td>
<td>OPA 15 - TTC CGA ACC C</td>
<td>4 μL DNA</td>
<td>5 μM dNTP</td>
<td>94°C – 1’</td>
<td>This study</td>
<td></td>
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**Note:** PCR profile for OPA14 and OPA15 is as per Table 1.
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RAPD for performance assessment of PALCAM and CHROMagar™ Listeria

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References


