Analysis of Antibiotic Susceptibility Profile and RAPD Typing of Listeria Monocytogenes Isolates

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Abstract
Background: The aim of the present study was to analyze antibiotic susceptibility profile and genetic diversity of L. monocytogenes, isolated from chicken carcasses using RAPD-PCR.
Methods: In this study, 26 isolates of Listeria monocytogenes recovered from chicken carcasses were used. Antibiotic susceptibility of these isolates were examined for eleven antimicrobial agents and RAPD-PCR with use of three different primers were applied to detect genetic diversity of the isolates.
Results: Some of the isolates showed multidrug resistance to commonly used antibiotics. Totally, 26 L. monocytogenes isolates had 16 different antibiogram patterns. Isolates of A and B clusters showed 7 and 6 different pattern, respectively. 12 Isolates of cluster C showed 7 different pattern.
Conclusions: Some of isolates had multidrug resistance to antibiotics. This may raise a public health hazard. RAPD-PCR showed that L. monocytogenes isolated from chicken carcasses had low genetic diversity and there was no clonal relationship between the isolates.
Keywords: L. monocytogenes; antibiotic resistance; RAPD-PCR

1. Introduction

The genus Listeria consists of a group of Gram-positive bacteria with low G + C content. Listeria species are non-spore forming bacilli found in different kinds of food and environment (1). L. monocytogenes causes a severe foodborne disease with a high mortality rate (20%) in humans. The main route of transmission of listeria to both humans and animals has been recognized as consumption of contaminated foods or feeds (2-3).

Different phenotypic and genotypic methods are used for characterization of Listeria monocytogenes isolates. Phenotypic methods as like as antibiogram (Antibiotic susceptibility) have low discriminatory power. Therefore, genotyping methods such as pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) (4) which have a higher discriminatory power have been employed.

In RAPD assay, characterization of genomic DNA is based on the number and the size of amplified DNA fragments generated by employing a single random or universal primer in a PCR. There are several advantages of RAPD over conventional PCR including, use of 8–10 base-pair (bp) length random sequence primers, without necessity of a prior knowledge of the target DNA sequence (5). Whereas for finger-printing of isolates, at least three universal primers in separate reactions are used, and subsequent banding patterns are compared either totally or for each primer. The superiority of RAPD typing compared with other molecular methods are based on several criteria such as, its simplicity, economy, fastness and high throughput nature (5).
The aim of the present study was to analyze the antibiotic susceptibility profile and determination of genetic diversity of L. monocytogenes isolates recovered from chicken carcasses using RAPD-PCR.

2. Methods

2.1. Bacteria
A total of 26 Listeria monocytogenes isolates were recovered from fresh chicken carcasses using enrichment in Listeria Enrichment Broth (LEB) and selective isolation on Oxford agar. They were confirmed by multiplex PCR (6). Antibiotic susceptibility and RAPD-PCR were performed on these 26 isolates.

2.2. Antimicrobial susceptibility test
The antimicrobial susceptibility profile of L. monocytogenes isolates were determined by use of standard disc-diffusion method recommended by CLSI (7) on Mueller-Hinton agar (Merck, Darmstadt, Germany). Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213 were used as reference strains. The following antimicrobial discs were used: Ampicillin (10 µg), Tetracycline (30 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Penicillin (10 U/IE), Chloramphenicol (30 µg), Gentamycin (10 µg), Vancomycin (30 µg), Trimethoprim (5 µg) and Rifampin (5 µg). The diameter of growth inhibition zone around each antimicrobial disc was measured after an incubation period of 24 h at 37 °C. The results were interpreted according to the CLSI (2013).

2.3. Random amplified polymorphic DNA analysis (RAPD)
The 26 isolates of L. monocytogenes were selected and analyzed using RAPD-PCR with three different primers, including D8635 (8), HLWL74 (9) and OPM01 (10). The sequence of these primers are shown in Table 1 and the amplification conditions are shown in Table 2. In each reaction 10 picomol of primer and 20 ng of extracted DNA were used. All reaction mixtures were carried out in a final volume of 25µl and amplified by a thermal cycler (Techne TC-512, UK).

Four microliter of each PCR product was electrophoresed in 1% agarose gel, which was prestained by ethidium bromide and visualized under UV illumination. All isolates were analyzed twice and controls were included in all the reactions to ensure reproducibility.

2.4. Interpretation of PCR fingerprint images
Photocap software was used to analyze scanned images. The data were analyzed using SPSS software, ver.16. Due to binarization of data, isolates were clustered and displayed in dendrogram form by using Jaccard distance matrix and Ward’s hierarchical cluster technique.

<table>
<thead>
<tr>
<th>Table 1: Sequence of used primers</th>
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</thead>
<tbody>
<tr>
<td><strong>Target gene</strong></td>
</tr>
<tr>
<td>D8635</td>
</tr>
<tr>
<td>HLWL74</td>
</tr>
<tr>
<td>OPM01</td>
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</table>

<table>
<thead>
<tr>
<th>Table 2. The amplification reaction of RAPD primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>D8635</td>
</tr>
<tr>
<td>HLWL74</td>
</tr>
<tr>
<td>OPM01</td>
</tr>
</tbody>
</table>
3. Results

3.1. Antimicrobial susceptibility of L. monocytogenes isolates

The resistance of L. monocytogenes isolates were tested to eleven antibiotic agents in this study. Seven isolates were sensitive to all antimicrobial agents. All of the L. monocytogenes isolates were sensitive to Ampicillin and Vancomycin. The most common finding was resistance to Erythromycin (61.53%), followed by resistance to Clindamycin (50%), Tetracycline (46.15%), and Trimethoprim (26.92%). Five (19.23%) isolates were showed resistance to Gentamycin and Chloramphenicol. Resistance to Ciprofloxacin and Rifampin were seen in four isolates (Table 3).

Table 3. Antimicrobial susceptibility profile of L. monocytogenes isolates

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Susceptible</th>
<th>Intermediate Resistance</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30.76%</td>
<td>23.07%</td>
<td>46.15%</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>34.61%</td>
<td>3.84%</td>
<td>61.53%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>73.07%</td>
<td>11.56%</td>
<td>15.38%</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>38.47%</td>
<td>11.53%</td>
<td>50%</td>
</tr>
<tr>
<td>Penicillin</td>
<td>92.30%</td>
<td>0%</td>
<td>7.70%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>76.92%</td>
<td>3.84%</td>
<td>19.23%</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>80.77%</td>
<td>0%</td>
<td>19.23%</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>57.69%</td>
<td>15.38%</td>
<td>26.92%</td>
</tr>
<tr>
<td>Rifampin</td>
<td>69.23%</td>
<td>15.38%</td>
<td>15.38%</td>
</tr>
</tbody>
</table>

3.2. Analysis of the amplified DNA polymorphisms

All primers worked well in amplifying multiple bands. D8635 primer amplified four different bands (650, 700, 1300 and 1500 base pair). Using this primer one polymorph pattern was generated. HLWL74 primer produced 17 different bands with sizes between 200 to 1800 bp.

Regarding OPM01 primer, 26 different bands with 190 bp to 2850 bp length were produced. In this primer reaction one polymorph band was produced.

Totally, 49 different bands with sizes ranging from 190 base pair (bp) to about 3850 bp were produced. Two bands were polymorph. According to our results, low polymorphism (4.08%) were detected among these isolates. In other words, genetic diversity was low among these 26 isolates. In our study, 47 monomorph bands were detected among 26 isolates of L. monocytogenes. No unique band was detected among these isolates.

Similarity matrix was constructed based on the presence or absence of a band for each isolate which was scored as 1 and 0, respectively. The biggest genetic similarity (86.7%) was seen between isolates no 13 and 15. Isolates No. 14 and 15 had about 80% genetic similarity. The lower similarity (70.6%) was observed between isolates No.13 and 14. Figure 1 shows the dendrogram of L. monocytogenes isolates obtained with three primer set. Listeria monocytogenes isolates showed four different clusters (designed as A to D). (Figure 1)

Twelve isolates (46.15%) displayed a single RAPD profile (cluster C). Isolates in this cluster produced 10 to 17 bands. The second most frequent RAPD profile named as cluster A, accounted for 26.92% of isolates (7 of 26 isolates). However, type A appeared to be more prevalent in two points of sampling area. Only, isolate 2 was grouped into cluster D. This isolate was sensitive to all antimicrobial agents. The remaining L. monocytogenes isolates (23.07%) were associated with cluster B (isolates 3, 5, 18, 19, 23 and 25). RAPD showed a reproducibility level of more than 95%, and control assays, which contained no cell lysate, yielded no detectable amplified product.

3.3. Analysis of Antibiogram and RAPD patterns of L. monocytogenes isolates

Totally, 16 different antibiogram PAPD patterns (1-16) were detected in 26 L. monocytogenes isolates. Isolates of A and B clusters showed 7 and 6 different pattern, respectively. 12 Isolates of cluster C showed 7 different pattern.
4. Discussion

Generally it is recommended that at least three random primers should be used if RAPD is the only molecular typing method that has been used (9). In this study the three random primers (OPM-01, HLWL 74, and D8635) were used. These primers have already shown their discriminatory power in other studies (8, 10).

The applicability of RAPD test for sub-typing of L. monocytogenes isolates has been documented in many studies from many different laboratories (11, 12). RAPD has been widely employed for typing isolates from poultry processing plants (11, 13). Minor changes of the genomic DNA will lead to different sizes and numbers of amplified fragments (5).

Although, in a study several isolates from different types of RTE foods were indistinguishable by REP-PCR, they were differentiated by BOX-PCR and RAPD (12).

In this study, RAPD analysis of 26 isolates of L. monocytogenes from chicken carcasses with three different primers revealed four different clusters (designated as A to D) among 26 isolates. Among these 26 isolates of L. monocytogenes, 16 different antibiogram patterns were observed.

Seven isolates of L. monocytogenes (8, 9, 11, 13, 14, 15 and 16) were grouped into Cluster A and they classified into 7 different antibiogram patterns. The most common feature of this cluster was resistant to Erythromycin. About 83.33% of the isolates of these cluster had resistant / intermediate resistant to Tetracycline and Clindamycin.

Similarity matrix was constructed based on the presence or absence of a band for each isolate which was scored as 1 and 0, respectively. The biggest genetic similarity (86.7%) was seen between isolates no 13 and 15, which were resistant to Clindamycin and Erythromycin. Isolates No. 14 and 15 had about 80% genetic similarities. Resistance to Erythromycin was their common feature (Isolate no. 14 & 15). Isolate no. 13 and 14 had about 70.6% similarities, both of these isolates were resistant to Erythromycin, but isolate No: 13 showed also resistance to Clindamycin and Tetracycline whereas isolate No: 14 had intermediate resistance to these antimicrobial agents. Jamali and Thong (12), reported that two sets of L. monocytogenes isolates that had identical pulsotypes were grouped into the same
cluster in RAPD and were clonally related (more than 80% similarity).

In this study, 6 isolates with different antibiogram patterns were associated with Cluster B. 5 of 6 isolates in these cluster were resistant to Tetracycline and Clindamycin. Although, 3 isolates of this cluster had some similarity in antibiogram patterns. But, 4 of them had resistance/intermediate resistance to five antibiotic agents including: Clindamycin, Tetracycline, Rifampin, Erythromycin and Trimethoprim. The isolates which were indistinguishable by both RAPD-PCR and antibiogram, were isolated from chicken carcasses in the same location and sampling time, strongly suggesting high prevalence of these isolates in the tested area.

Cluster C consisted of 12 isolates of L. monocytogenes and was the predominant cluster. Isolates which were grouped in this cluster produced 10 to 17 bands and had a heterogeneous antibiogram pattern. 7 different antibiogram patterns were distinguished among them. 5 isolates (4, 17, 21, 22, and 26) were totally sensitive to antimicrobial agents. 5 isolates (1, 6, 7, 20 and 24) had resistance to four antimicrobial agents (CC, TE, E, and TMP). Isolate No: 10 only had resistance to Tetracycline and sensitive to 10 other antimicrobial agents. But isolate No: 12 had resistance to Penicillin, Erythromycin and Trimethoprim. However, resistance to Erythromycin and Trimethoprim in isolate No: 12 were similar to other five isolates mentioned above.

Cluster D with one isolate was the last cluster. This isolate was sensitive to all antimicrobial agents.

Genotyping by RAPD-PCR showed that the chicken L. monocytogenes isolates were diverse and heterogeneous. Since the isolates were from different areas and various sampling times, such heterogeneity might be due to environmental contamination of chicken carcasses. Also, there was low correlation between the antibiogram and RAPD-types. Similar observation were reported in different kinds of food’s L. monocytogenes isolates (13-17). This is expected because genotyping methods detect variations of the bacterial genome, whereas most antimicrobial-resistance genes are carried on plasmids (14). Purwati et al. (18) observed 27 RAPD types in 28 L. monocytogenes isolates recovered from chicken meat. These isolates showed resistance to three or more antimicrobial agents. They concluded that RAPD sensitivity was higher than antibiogram.

Adzitey et al (11) reported that three antibiotic patterns, 5 clusters and 3 singletons were seen in fifteen isolates of L. monocytogenes. So, RAPD had a higher power in distinguishing of isolates.

Some isolates with the same RAPD pattern exhibited different antimicrobial resistance profiles. The observed lack of association could be due to the small sample size. Hence, for better understanding, a bigger sample size from wide geographical areas and different kinds of foods should be investigated in future studies.

5. Conclusion

In conclusion, presences of multidrug resistance of L. monocytogenes in the chicken meat indicate the potential risk of infection with these bacteria, especially among peoples consuming chicken meat barbecue, which is a popular Iranian meal.

Genotyping data showed that the chicken L. monocytogenes isolates were not so genetically diverse and heterogeneous. Different methods have different levels of discriminatory powers; so the application of more than one subtyping approach would provide a more accurate picture of the relatedness of L. monocytogenes isolates.

5.1. Compliance with Ethical Standards

The authors declared that there is no conflict of interest regarding the results of the present study. Also, no human participants or animals involved in this study.

References


