Background: Listeria monocytogenes, as one of the foodborne pathogens, is a causative agent of listeriosis. The transfer of L. monocytogenes bacterium in pregnant women occurs as self-limited flu-like symptoms, but it may result in abortion, stillbirth or premature birth of the infected baby. One of the best methods for detection of this bacterium is polymerase chain reaction (PCR).

Objectives: The purpose of this study was detection of virulence factors (hlyA and plcA) of L. monocytogenes in women with abortion, using PCR.

Patients and Methods: In this pilot and cross-sectional study, 96 patients with abortion admitted in educational university, Tehran, Iran were surveyed for L. monocytogenes by PCR and culture methods. Some variants like age, occupation, history of abortion and education were considered for all patients. Vaginal swabs and secretions were transferred to transport media and then all the samples were transferred to a microbiology laboratory. The tubes were incubated in 4 ºC and the specimens were cultured on PALCAM media. The isolates were verified by Gram staining, catalase and oxidase test, methyl red-Voges-Proskauer (MR-VP), sugar fermentations and motility in 20-25 ºC. Then, PCR was performed for the extracted DNAs. Data were analyzed by SPSS software version 17, and χ² (Chi-square test).

Results: Out of 96 samples, 16 isolates of L. monocytogenes by PCR (plcA and hlyA) and four isolates by culture were identified. There was a significant difference between PCR and culture methods (\( P = 0.003 \)). The results of this study showed that PCR was more sensitive and specific than culture method. There was also a significant association between the bacteria and hlyA and plcA genes and human abortion and between patients with abortion precedence and education.

Conclusions: Based on our study, plcA and hlyA played a key role in the virulence determination of L. monocytogenes. Data analysis also showed that L. monocytogenes could be a causative agent of abortion in pregnant women.

Keywords: Listeria monocytogenes; Abortion; Polymerase Chain Reaction; Culture

1. Background

Listeria monocytogenes, a bacterium that causes foodborne infections, can result in abortion and diseases as severe as encephalomalagnitis, septicemias and gastroenteritis. It is a facultative intracellular and Gram-positive bacterium, presented in pregnant women by flu-like symptoms (1-3). Along with the occurrence of listeriosis in adults, it is especially important in fetuses, since around 40% of all cases are pregnancy-related, where the disease can cause miscarriage, premature birth, stillbirth, and neonatal disease (2, 4). The incidence of listeriosis among pregnant women is 17 times as high as the general population (12 per 100 000 vs. 0.7 per 100 000, respectively) (5).

L. monocytogenes has six basic virulence genes including prfA, plcA, hlyA, mpl, actA, and plcB, located together in one virulence gene cluster between the house keeping genes idh and prs (6). L. monocytogenes is identified by selective enrichments and biochemical analyses. One of the best instruments that has a tremendous potential for detection of foodborne pathogens is polymerase chain reaction (PCR), and therefore it has been one of the most sought-after methods in food microbiology in recent years (7-9).

2. Objectives

The objective of the present study was detecting the virulence-associated gene(s) in L. monocytogenes by PCR and culture methods among women with abortion. Finally, we compared the two results.

3. Patients and Methods

3.1. Samples Collection

In this pilot and cross-sectional study, a total of 96 samples including vaginal swabs were collected by convenience samplings method from patients with sponta-
nenous abortions who had been admitted to educational medical centers of Tehran, Iran, during June 2012 to May 2013. All the specimens were transferred on ice to the Microbiology Research Laboratory of Shahid Beheshti University of Medical Sciences.

3.2. Inclusion and Exclusion Criteria

Inclusion criteria was the swab of a sample of abortion under 20 weeks of pregnancy, and exclusion criteria was the swab of a sample of pregnancies more than 20 weeks of pregnancy.

3.3. Enrichment, Culturing, and Morphological and Biochemical Identification

Briefly, the vaginal swabs were placed in 10 mL tryptic soy broth with yeast extract (TSBYE) the samples were incubated at 4°C. After a one-week period or a one to three month period of incubation, aliquots from the enrichment broth (TSBYE) were streaked onto PALCAM agar (Merck, Germany) and blood agar (Merck, Germany) and the plates were incubated at 37°C for 24 to 48 hours. Green shiny colonies with diffused black shadows around them on PALCAM agar and yellow small colonies with ß-hemolysis on blood agar were suspected to be Listeria. The isolates were confirmed by Gram staining, catalase reaction, oxidase test, motility at 20-25°C, methyl red-Voges Proskauer (MR-VP) fermentations, and tests of sugars (xylose, rhamnose and mannitol). All the biochemically characterized isolates were tested for haemolytic on sheep blood agar.

3.4. DNA Extraction

DNA extraction was performed by AccuPrep genomic DNA extraction kit (Bioneer Co., Korea). After one week, the vaginal swab specimens with TSBYE were incubated at 37°C for 24 to 48 hours. Green shiny colonies with diffused black shadows around them on PALCAM agar were tested for haemolysis on sheep blood agar.

3.5. Polymerase Chain Reaction

The PCR was standardized for detecting two virulence associated genes of L. monocytogenes, namely plcA and hlyA, to 659 and 451 bp PCR products respectively. Among 96 samples, L. monocytogenes was found in 16 samples entailing plcA and hlyA genes according to the PCR results (Figures 1 and 2).

3.6. Data Analysis

All data were compiled, and analysis was done using ssp version 17 and for survey of significance, Chi-square test was calculated. Minimal level of significance was considered P < 0.05.

4. Results

Among 96 samples from spontaneous abortions, four specimens were positive for L. monocytogenes by microbiological tests. The standardized PCR allowed amplification of virulence-associated genes of L. monocytogenes, namely plcA and hlyA, to 659 and 451 bp PCR products respectively. Among 96 samples, L. monocytogenes was found in 16 samples entailing plcA and hlyA genes according to the PCR results (Figures 1 and 2).

Statistically, there was a significant difference between these two methods (P = 0.003). This study showed that PCR was more sensitive and specific than culture method.

The average age of the 96 women was 30.9 ± 4.7 years old and the average age of patients with positive L. monocytogenes PCR results was 33.5 ± 7.2 years old. Of 96 samples, 27 patients (28.1%) were low educated, 53 (55.2%) had a high school diploma and 16 (16.7%) had a college degree. High school graduates and low-educated patients had the highest incidence of Listeria compared with higher educated ones (L = 68.84). Therefore, there was a significant association between patients positive for Listeria and control samples regarding education; 95% CI [2.3-85.7 OR=12.2] (P = 0.00).

Analysis of all the samples indicates that of 16 patients positive for L. monocytogenes, 6 (37.5%) had abortion precedent. There was a significant association between patients with abortion precedent and with non-abortion precedent; 95% CI [1.7-33.5 OR=74] (P = 0.001).

Analysis of all the samples also showed that there was no significant association between women with positive and negative Listeria regarding the contraception methods (Table I). We did not encounter any missing value, either.

### Table I. Frequency of Listeria monocytogenes Based on the Research Variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Prevalence of L. monocytogenes by PCR</th>
<th>Prevalence of L. monocytogenes by Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Low level of education</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>21-30 Years</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>31-41 Years</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Higher than diploma</td>
<td>2</td>
</tr>
<tr>
<td>Education</td>
<td>Total</td>
<td>16</td>
</tr>
<tr>
<td>Age</td>
<td>Lower than 20 years</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>21-30 Years</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>31-41 Years</td>
<td>3</td>
</tr>
<tr>
<td>Age</td>
<td>Total</td>
<td>16</td>
</tr>
<tr>
<td>Employment</td>
<td>Non-abortion Precedence</td>
<td>6</td>
</tr>
<tr>
<td>Job</td>
<td>Total</td>
<td>16</td>
</tr>
<tr>
<td>Contraception methods</td>
<td>Natural</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Other methods</td>
<td>9</td>
</tr>
<tr>
<td>Job</td>
<td>Total</td>
<td>16</td>
</tr>
</tbody>
</table>

a. Abbreviation: PCR, polymerase chain reaction.

b. Data are presented as No.

Figure 1. Northern Blot Analysis of L. monocytogenes DNA with phylogenetic probes. Lane M, phage marker; Lane 1, positive control; lanes 2-8, positive samples; lane 9, negative control. Lane M, 100-bp PCR marker.
5. Discussion
Few human listeriosis cases have been reported in Iran. This can be due to imperfect identification or isolation methods, lack of awareness, and low incidence rate in some regions. Approximately, one-third of reported human listeriosis is associated with pregnancy, causing spontaneous abortions, especially in the second or third trimester (10).

In the present study, 16 and four isolates of L. monocytogenes were recovered from human abortion cases by PCR and culture, respectively. The rate of L. monocytogenes identified by PCR was 16.7%. The result of the used methods as well as the data analysis showed that L. monocytogenes can be a causative agent of abortion in pregnant women.

A number of factors are involved in manifestation of L. monocytogenes virulence. Detection of only one virulence-associated gene by PCR is not always sufficient to identify L. monocytogenes (11, 12). Moreover, two genes (plcA and hlyA) were investigated in our study. L. monocytogenes phospholipases are essential determinants of pathogenicity and thus, we investigated them as well (5), plcA and hlyA existed in all the positive cases (16).

In a study by Kaur et al. the prevalence of L. monocytogenes in India reported four isolates from 305 samples comprising blood, urine, and placental bits, faecal and vaginal swabs that were collected from 61 patients with spontaneous abortion samples. Another result of this research was that plcA and its expression had key roles in virulence determination of L. monocytogenes (5).

Shayan et al. reported 36 isolates of L. monocytogenes in Iran with PCR method and 7 isolates with culture, among 100 vaginal samples. In accordance with this study, PCR was faster, more sensitive and more specific than culture method for identifying L. monocytogenes in vaginal swabs. Because of some factors like the number of samples and country similarity, the results of this research were similar to our results (13).

In a research study conducted by Kargar et al. in Iran, out of 311 samples (urine, blood, placenta and cervix), 12 were detected for L. monocytogenes using both culture and PCR methods. The results also showed a significant statistical relationship between recurrent abortions and level of education using PCR (P < 0.05). Because of several factors like age, positive cases and education, the results of this study and our study had more similarity compared to other researches (18).

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Authors’ Contributions
The core idea of this work was from Gita Eslami who also conducted the project as part of her dissertation. Elahe Chavoshi Shayan provided the specimens and the data, cultured and performed the molecular tests, and wrote the manuscript with the assistance of Arefe Zarehpoor, Hossein Goudarzi and Arefe Sahperbour were the advisors of this study. Bita Pourkaveh contributed in data collection and manuscript writing.

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