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Pregnancy-related listeriosis: frequency and genotypic characteristics of *L. monocytogenes* from human specimens in Kerman, Iran

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Summary *Listeria monocytogenes* is a foodborne pathogen that can pose serious complications during pregnancy and neonatal infection. This study aimed to determine the frequency of *L. monocytogenes* infection, prevalent serotypes, and virulence genes among pregnant women and those experiencing miscarriages in Kerman, Iran. Out of 200 vaginal swabs, 4.5 and 29.5% of specimens were positive for *L. monocytogenes* infection as identified by culture and molecular methods, respectively. The majority of isolates from positive cultures (89%) of pregnant women resulted in stillbirth, death, and blindness. The most prevalent virulence determinants were *inl* B, *prf* A, and *act* A. The majority of isolates were non-typable. A history of miscarriage and gestational age are known to be significantly associated with the presence of infection. This study emphasizes the importance of initial screening for *L. monocytogenes* in pregnant women in Iran. Molecular methods may be useful in this process. Increasing the awareness of pregnant women could be effective in reducing pregnancy-related listeriosis.

Keywords *Listeria monocytogenes* · Miscarriage · Pregnancy-related listeriosis · Vaginal swab · Iran

Introduction

Listeria monocytogenes is an aerobic gram-positive bacillus which can be isolated from sources such as foods of animal origin, vegetables, and from the feces of mammals, birds, and other animals. The main route of infection by this organism is through ingestion of contaminated food, such as unpasteurized milk and raw unwashed vegetables; however, transmission from mother to child can also occur in utero or at birth [1, 2].

Due to intracellular localization of *L. monocytogenes* and decreased cell-mediated immunity in pregnancy and the neonatal period, a marked association of infections with pregnancy or the neonatal period has been demonstrated [3, 4]. Currently, the bacterium is best recognized for its ability to colonize and infect the placenta of pregnant woman, subsequently causing fetal infections or miscarriage [5]. It is believed that pregnant women are 18-times more prone to listeriosis than non-pregnant women [5, 6]. According to a report by the World Health Organization, pregnancy-related listeriosis accounts for about 43% of all listeria infections, of which approximately 14% occur during the third trimester of pregnancy [4, 6].

In Listeria spp., the *prf* A gene is the identified positive regulatory factor for a cluster of virulence genes such as factors for attachment (internalins), listeriolysin O, two phospholipase C enzymes, invasion-associated protein (Iap), and a protein mediating actin-directed intracellular motility (act A). Internalins (InlA and InlB) are surface-associated proteins mediating the entry of bacteria into mammalian cells. Listeriolysin O (LLO) encoded by *hly* A is a cholesterol-dependent toxin which contributes to bacterial escape from phagocytic vacuoles and replication in the host cytosol. Act A is another surface protein that mediates the intra-cytoplasmic movement of bacteria by polymerization of actin [6–9].

Strains of *L. monocytogenes* form a structural population of divergent lineages. Thus far, four lineages (I–IV) have been identified for *L. monocytogenes*. Lineage I (serotypes 1/2 b, 4 b) and lineage II

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Table 1 Primers used for detection of virulence genes

Target gene	Primer sequence (5′–3′)	Ampliqon size (bp)	Reference
act A	F:AACACAGATGAATGGGAAGAAG R: TCCACTTGTATAGCTGGTCG	278	[15]
inl B	F:TGATGCTTTTGCAGAAACAATC R:ATCACTTATACCATTATGCTCC	319	[15]
prf A	F:TCACGAGTATTAGCGAGAACG R: TAGCTAGACTCTATCAAACTTG	246	[29]
hly A	F: CATTAGTGGAAAGATGGAATG R: GTATCCTCCAGAGTGATCGA	730	[30]

R reverse, *F* forward, *bp* base pairs

(serotype 1/2 a) are responsible for most of the infections in humans [10, 11].

Because no valid databases for listeriosis are available and the epidemiology of pregnancy-associated cases is unknown in Iran, estimation of the disease load in the Iranian population seems to be required. For this purpose, the current study was performed to determine the frequency, prevalent serotypes, and virulence genes of *L. monocytogenes* isolated from pregnant women and those having miscarriages in this region.

Materials and methods

Study population

In this cross-sectional study performed from December 2015 to December 2016, a total of 200 vaginal swabs were taken from 124 women who had experienced miscarriages and 76 pregnant women (aged 18–42 years) referring to or hospitalized at the gynecology department of a university-affiliated hospital in Kerman. The study-site hospital is a 462-bed tertiary care teaching hospital and is a major university hospital in southeastern Iran. The gynecology department of the hospital consists of delivery, perinatology, and in vitro fertilization (IVF) sections. Approximately 8000 pregnant women refer yearly to this department.

All volunteers completed a questionnaire containing demographic data (age, education, place of residence, etc.) and information such as history of miscarriage, premature delivery, genital infection, and urinary tract infection under the supervision of a gynecologist. Any history of consumption of unpasteurized dairy products, the method of washing vegetables and processing the meat were asked through face-to-face interviews by the researcher. Patients diagnosed with immunodeficiency, chronic diseases such as diabetes, endocrine disorders, and hypertension were excluded from the study. All women signed informed consent forms before participating in the study.

Specimen collection was performed by inserting and rotating 2–3 cm of a Dacron swab (Delta, Spain) into the vagina to obtain a large number of cells. All specimens were placed into trypticase soy broth enriched with 0.6% yeast extract (TSBYE) and were transferred on ice to the microbiology laboratory for further processing [12].

L. monocytogenes (ATCC 7644) was used as the positive control for all experiments [13]. Amplification of one or more virulence genes was considered to confirm the presence of *L. monocytogenes* [14].

Detection of virulence genes and serotyping by molecular methods

The polymerase chain reaction (PCR) mixture was prepared for amplification of all the genes with a total volume of 25 µl consisting of 2 µl of bacterial DNA, 0.2 µl of each specific primer, and 13 µl of 2 × Master Mix Red (Ampliqon, Odense, Denmark). The master mix consisted of 0.2 unit/µL of Ampliqon Taq DNA polymerase, 1.5 mM $MgCl_2$, and 0.4 µM dNTPs [15]. The specificity of all primers was verified using Primer Quest software (http://www.ncbi.nlm.nih.gov/Gene). The primer sequences and amplicon size of PCR products are shown in Table 1.

The genotypes of the isolates were determined using sets of primers D1 and D2 and were further subtyped using FlaA and GLT primers as indicated in Table 2 [11]. *L. monocytogenes* ATCC 7644 (Division I) and *L. monocytogenes* ATCC 35,152 (Division II) were used as positive controls.

Statistical methods

Statistical analysis was performed using SPSS (Version 20; SPSS, Inc) software. The chi-square test was used to find significant correlations between characteristics of patients and detection of *L. monocytogenes*. A p-value < 0.05 was considered statistically significant.

Results

Prevalence of L. monocytogenes in study groups

Using conventional methods, *L. monocytogenes* was isolated from nine women (9/200; 4.5%) including eight pregnant women (8/76; 10.5%) and one woman who had experienced miscarriage (1/124; 0.8%). Using the PCR method, 59 specimens (59/200; 29.5%) were shown to be positive for *L. monocytogenes*, comprising 31 isolates from the miscarriage group (31/124; 25%) and 28 isolates from the pregnant group (28/76; 36.8%). Overall, 42.5% of the study population had a history of miscarriage. Although no association was found between the history of miscarriage and detection of *L. monocytogenes* in infected groups (pregnant and miscarriage), there was a significant difference between infected and non-infected groups ($p < 0.05$). A history of one or more miscarriages was significantly higher in the infected groups (54.5 versus 37%). Also, *inl* B and *act* A were the most prevalent detected genes in these specimens.

Table 2 Primers and PCR condition for molecular serotyping of *L. monocytogenes*

Primer set	Primer sequence (5′–3′)	Product size (bp)	Annealing Temperature (°C)	Specificity
D1	F:CGATATTTTATCTACTTTGTCA R:TTGCTCCAAAGCAGGGCAT	214	59	Division I or III
D2	F:GCGGAGAAAGCTATCGCA R:TTGTTCAAACATAGGGCTA	140	59	Division II
FlaA	F:TTAACTAGATCAAACTGCTCC R:AAGAAAAGCCCC TCGTCC	538	59	Serotype 1/2a and 3a
GLT	F:AAAGTGAGTTCTTACGAGATTT R:AATTAGGAAATCGACCTTCT	483	55	Serotype 1/2b and 3b

R reverse, *F* forward, *bp* base pairs

Table 3 Frequency of virulence genes in *L. monocytogenes* in women from miscarriage and pregnant groups identified by culture and PCR assay

Virulence genes	Miscarriage group *N* (%)		Pregnant group *N* (%)	
	Culture *N* = 1	PCR *N* = 31	Culture *N* = 8	PCR *N* = 28
act A	0	12 (38.7)	6 (75)	16 (57.14)
inl B	1(100)	9 (29.03)	7 (87.5)	19 (67.85)
Prf A	0	15 (48.38)	7 (87.5)	13 (46.42)
hly A	0	2 (6.45)	1 (12.5)	1 (3.58)
act A+ *inl* B	0	2 (6.45)	6 (75)	10 (35.72)
act A+ *Prf* A	0	3 (9.68)	6 (75)	8 (28.58)
inl B+ *Prf* A	0	0	6 (75)	9 (32.15)
act A +*inl* B +*Prf* A	0	0	6 (75)	7 (25)
hly A+ *act* A	0	1(3.23)	0	0
hly A +*inl* B	0	0	0	1(3.58)
hly A +*prf* A	0	2(6.45)	0	0

A significant association was found between the gestational age of pregnancy and the presence of *L. monocytogenes* ($p \leq 0.05$). So that 61.2% (19/31) of infected specimens in the miscarriage group were isolated from women at 11–20 weeks of gestational age, while only 3.5% (1/28) of infected specimens in the pregnant group were from women at these weeks.

PCR analysis of all recovered isolates revealed variable genotypic patterns for virulence-associated genes: *inl* B, *act* A, *hly* A, and *prf* A. The *prf* A gene was the most frequent in the miscarriage group (Table 3).

Molecular serotyping

The majority of culture-positive isolates (6/9, 66.6%) were non-typable. Serotypes 4 and 1/2 c were detected separately in two isolates. In 59 PCR-positive specimens, non-typable isolates were most frequently followed by serotypes 1/2 c, 3 c (7/59, 11.9%), 1/2 a, 3a (3/59, 5.1%), and serotype 4 (4/59, 6.8%; Table 4). Follow-up studies showed that two of the nine women infected with *L. monocytogenes* (identified by culture) experienced stillborn delivery. Additionally, the infants of two mothers died after 1 week of hospitalization at NICU (Table 5).

Discussion

L. monocytogenes is the etiological agent of listeriosis, an infectious disease with a mortality rate of 20 to 30% in certain risk groups. Apart from high mortality, the severity of human listeriosis makes this infection a major public health concern. Pregnancy seems to be the most common risk factor and infected women are at risk of miscarriage, stillbirth, and premature labor. However, pregnancy-associated listeriosis has emerged as an asymptomatic disease with no specific clinical symptoms, which raises challenges to its diagnosis.

Although some reports exist about listeriosis among pregnant women globally, comparison with the current results is difficult because of differences in the parameters which can affect the results. These include an understudied population (socioeconomic status, nutritional habits, etc.), types of specimens (vaginal swabs, rectal swabs, placental bits, urine, etc.), and detection methods (culture, molecular method, etc.).

Although, culture is the gold standard for identification of *L. monocytogenes*, it appears that molecular detection can be a valuable method during screening [16–18]. In the current study and those conducted in Iran and Egypt, positive results from molecular methods were higher than those from culturing [15, 18, 19]. A variety of virulence genes have been proposed for

Table 4 Frequency of serogroups in *L. monocytogenes* in specimens from miscarriage and pregnant women identified by culture and PCR assay

Serogroups	Miscarriage group *N* (%)		Pregnant group *N* (%)	
	Culture *N* = 1	PCR *N* = 31	Culture *N* = 8	PCR *N* = 28
1/2c, 3 c	0	3(9.67)	1(12.5)	4(14.28)
½ a, 3a	0	2(6.45)	0	1(3.58)
4	0	2(6.45)	1(12.5)	2(7.14)
Non-typable	1(100)	24(77.4)	0	21(75)

Table 5 Molecular characteristics of *L. monocytogenes* identified by conventional methods and follow-up study of infected women

Isolate code	Study group	Virulence gene	Serotype	Outcome of pregnancy
33	Pregnant	*act* A, *prf* A, *inl* B	4	Still birth
34	Pregnant	*act* A, *prf* A, *inl* B	NT	Live, healthy birth
38	Pregnant	*act* A, *prf* A, *inl* B	NT	Death in one of twins, live but blindness in the other
39	Pregnant	*act* A, *prf* A, *inl* B	NT	Live, healthy birth
44	Pregnant	*act* A, *prf* A, *inl* B	NT	Still birth
47	Pregnant	*act* A, *prf* A, *inl* B	NT	Live, healthy birth
58	Pregnant	*prf* A	NT	No information
72	Pregnant	*inl* B, *hly* A	1/2c	Death in one of twins, blindness in the other
90	Miscarriage	*inl* B	NT	Abortion

NT non-typable

molecular identification. In some studies, the presence of *act* A and internalin-encoding genes has been considered [6, 20]. In other studies, *prf* A and *hly* A are crucial to identification of *L. monocytogenes* [18, 21]. In the current study, *L. monocytogenes* was isolated from 9 specimens by culture, although 59 specimens had at least one of the above-mentioned genes.

Although different types of samples may be used for isolation of *L. monocytogenes* from clinical samples, some studies have reported that vaginal swabs are best for *L. monocytogenes* detection [16, 22]. This method was used in the current study.

The percentage of pregnancy-related listeriosis by the culture method in the current study (9/200; 4.5%) was lower than from two previous reports among women with miscarriages in Tehran, Iran (9%; 7.2%), India (6.5%), and the prevalence of pregnancy-related listeriosis in England and Wales (15%), but was higher than for pregnancy-related listeriosis in another studies in India (0.8%) [5, 6, 12, 15, 20].

The current results show that *inl* B was the most prevalent gene, perhaps because *inl* B is responsible for entry of the microorganism into a variety of cell types. Despite the similar pattern of virulence determinants in most of the identified isolates, the observation of a few differences in the virulence gene pattern confirms that there was heterogeneity in the virulence of strains.

In the current study, infection with *L. monocytogenes* had a significant relationship with a history of miscarriage ($p \leq 0.05$). Earlier studies from India indicated that the prevalence of *L. monocytogenes* in women with poor obstetric history was 1.34 to 14% [14] and in Iran was 5 to 7% [17]. The highest rate of *L. monocytogenes* infection among the miscarriage group in the current study was at 11–20 weeks of gestational age. Among pregnant women, this was more than 31 weeks. A significant association was found between the gestational age of pregnancy and the presence of *L. monocytogenes* ($p \leq 0.05$). This is similar to a report by Awofisayo et al. showing that most *L. monocytogenes* infection occurs in the second trimester and shows a significant relationship to gestational age [5].

The rate of isolation of *L. monocytogenes* from pregnant women in the current study was found to be higher than for women with miscarriages, which could be due to factors such as the time of sampling, which was at the time of delivery in the pregnant group and after miscarriage in the miscarriage group. The other factor was the type of sample, which was vaginal swab in both groups. In the latter group (miscarriage group), bacteria could have moved from the vagina to the fetus to have caused a miscarriage. Thus, this may be the cause of lower isolation of bacteria in this group.

Although most of the infected women declared they did not use a disinfecting agent when washing vegetables, no significant relationship was found between infected and non-infected groups, which is in contrast to the findings of Pourkaveh et al. [23]. These researchers also found that parameters such as age, mother's and father's educational levels, consumption of unpasteurized dairy products, soft cheese, improperly cooked meat, smoked meat, and sea food and a history of contact with soil and domestic animals were significantly associated with the presence of *L. monocytogenes* [23]. Consequently, infection with *L. monocytogenes* is complicated and multiple factors may be involved in the process.

Molecular serotyping

Typing of *L. monocytogenes* is the main route in epidemiological studies for investigation of foodborne outbreaks and helps in identification of the contamination source. To this purpose, PCR serotyping has been developed to separate the four major *L. monocytogenes* serovars (1/2 a, 1/2 b, 1/2 c and 4 b), with the serotype 4 b as the most frequent cause of human epidemics [24, 25]. In the present study, non-typable isolates were the most frequent, and serovars 1/2 c and 4 accounted for most major typable isolates. In Sweden, serotype 4 b was reported in 18% of human isolates [26]. In southern Spain, from 154 cases of human listeriosis from 2005 to 2009, the identified serotypes were 4 b (94; 61%), 1/2 b (30; 19%), 1/2 a (27; 18%), and 1/2 c (3; 2%) [27]. In another study, genotypic analysis of 17 *L. monocytogenes* isolates showed that 88.24% (15/17) of isolates belonged to serovar group 4 and 11.76% (2/17) to serovars 1/2 b and 3 b [25]. In another report, the prevalence of serotypes 4 b and 1/2 were similar, but 1/2 b had a lower prevalence [28].

Regarding culture-positive isolates in the current study, only one isolate having serotype 4 b was detected. Follow-up showed that the outcome of the pregnancy was stillbirth. However, the presence of other infectious agents could be responsible for such an outcome, including infections with viruses, chlamydia, and mycoplasma. Again, in the current study, one woman infected with the non-typable isolate experienced stillbirth; however, further studies are required to relate the stillbirth to infection with a specific serotype or virulence factors.

In conclusion, in the present study, the rate of isolation of *L. monocytogenes* from pregnant women having normal deliveries was higher than that in women experiencing miscarriages, although the presence of *L. monocytogenes* was found to be associated with a previous history of miscarriage. This study provides information about the status of pregnancy-related listeriosis in Kerman and emphasizes the importance of initial screening for *L. monocytogenes* infection in this region. As listeriosis is not a reportable disease in Iran, routine screening for the prevalence of *L. monocytogenes* would be useful to better understand the epidemiology of the organism and may be helpful in prevention of emerging cases. Epidemiological studies would help to identify the sources of infection and their risk factors, routes of transmission, and clinical forms, and allow for better management of listerial infection, especially in pregnant women.

Conflict of interest Z. Zahirnia, S. Mansouri, and F. Saffari declare that they have no competing interests.

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