



Contents lists available at ScienceDirect

Journal of Acute Disease

journal homepage: www.jadweb.org



Document heading doi:

Genotypic characterization, invasion index and antimicrobial resistance pattern in *Listeria monocytogenes* strains isolated from clinical samples

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ARTICLE INFO

Article history:

Received 9 February 2015

Received in revised form 11 February 2015

Accepted 12 February 2015

Available online 13 February 2015

Keywords:

Genotypic characterization

Invasion index

Antimicrobial resistance

Listeria monocytogenes

ABSTRACT

Objective: To evaluate antimicrobial resistance, invasion index and genetic profile in *Listeria monocytogenes* isolated from clinical samples. **Methods:** At all, 170 clinical samples were collected from patients with spontaneous abortions hospitalized in Shariati hospital in Tehran during June 2010 to August 2013. Invasion index was determined using HeLa cells. The multiple-locus variable-number tandem-repeats analysis (MLVA) was used for evaluation of genetic relatedness. **Results:** Out of 14 *L. monocytogenes* isolates, 4 (28.57%), 2 (14.28%), 0 (0%), 5 (35.71%) and 3 (21.42%) were isolated from placental tissue, urine, blood, vaginal and rectal swabs, respectively. High resistance to penicillin and multidrug resistant were found amongst isolates. The invasion index was in the range of 0.001–0.007. Seven different types were obtained by MLVA assay and type 2 and 3 with 4 strains were the most frequent type. Strains isolated from the vagina and the placenta of the same type were also more resistant to penicillin. **Conclusions:** Since MLVA is a high-throughput screening method that is fairly inexpensive, easy to accomplish, rapid, and trustworthy, it is well suited to interlaboratory comparisons during epidemiological investigations. Also further studies of larger samples from a variety of sources such as food and animal specimens recommended comparing by MLVA method.

1. Introduction

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacteria that is ubiquitous in soil, vegetable and other ecological sources[1–4], and causes materno-fetal infections, gastroenteritis and meningoenphalitis through its capability to cross the gastric, placental and blood-brain barriers, respectively[5]. Infection of pregnant

women is significant because placenta is a proper niche for *L. monocytogenes* growth[6]. According to Food Net US, listeriosis was responsible for 30% of foodborne deaths from 1996 to 2005 and had a high mortality rate to 16.9%[7].

Although most isolates are susceptible to antibiotics, recently, several cases of antibiotic resistance has been reported[8]. Therefore, perform of the antimicrobial susceptibility test is essential for *L. monocytogenes* isolates[9]. Several laboratory tests have been improved for virulence determination of *L. monocytogenes*. These include *in vitro* cell assays, *in vivo* bioassays and methods for main virulence related proteins and their responsible genes[10]. The severity of disease caused by *L. monocytogenes* makes it essential to use of a technique that can quickly detect outbreaks and be used in identification of source and tracking. Serotyping, ribotyping, Amplified fragment length polymorphism and pulsed field gel electrophoresis were used previously for typing of *L. monocytogenes*. Although

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pulsed field gel electrophoresis has a high discriminatory power, the method is time consuming and expensive and it is also difficult to interpret^[11,12]. In recent years, multiple-locus variable-number tandem-repeats analysis (MLVA) with agarose-gel separation has been described with good results^[13–16]. The aim of this study is an evaluation of antimicrobial resistance, invasion index and genetic profile in *Listeria monocytogenes* isolated from clinical samples.

2. Material and methods

2.1. Bacterial isolation

During June 2010–August 2013 a total of 170 clinical samples were collected from a hospitalized woman with spontaneous abortion in Shariati hospital in Tehran, Iran.

2.2. Enrichment, culturing, morphological and biochemical identification

Firstly, 5 mL from each of urine and blood samples and 20 g of the placental tissue were inoculated in 50 and 230 mL of TSBYE (Tryptic soy broth plus 0.6% yeast extract, Merck, Germany). Also, every of fecal and vaginal swabs were inoculated into 10 mL of TSBYE. Then, all the samples were incubated at 4 °C. After 2 weeks to 6 months incubation, aliquots from enrichment broth (TSBYE) were streaked on PALKAM Agar (Merck, Germany) and plates were incubated in 35 °C for 24 to 48 h. The green shiny colonies with diffuse dark shadow around them on PALKAM agar were doubted to be *Listeria*. Five typical colonies of *Listeria* were purified on Brain Heart Infusion agar (Merck, Germany) and recognized by morphological, cultural and biochemical tests. Approval of isolates was done by Gram's staining, oxidase test, catalase reaction, voges proskauer (MR–VP), methyl red tests and fermentation of sugars (rhamnose, xylose, methyl α -D-mannopyranoside and mannitol). All the biochemically characterized isolates were tested for haemolysis on Sheep Blood Agar, Christie, Atkins and Munch Petersen (CAMP) test. The definite isolates as *L. monocytogenes* were stored at preservation medium including glycerol (15%) and pepton (1%).

2.3. Antibiotic susceptibility test

The susceptibility of isolates were established by the disk diffusion agar method as approved by CLSI guidelines M45–A2 for *L. monocytogenes* strains^[17], and eight antibiotic discs, namely chloramphenicol (10 μ g), penicillin G (10 U), tetracycline (25 μ g), streptomycin (10 μ g), trimethoprim (5 μ g), ampicillin (10 μ g), erythromycin (15 μ g) ciprofloxacin (5 μ g), (Himedia, India) were used. Also, *L. monocytogenes* ATCC 7644 was used as the reference strain.

2.4. Invasion index determination

To assess the invasiveness of strains the HeLa cells were

used. Strains were inoculated to the brain hearts infusion broth and then incubated at 37 °C for 24–48 h. Optical density was determined by spectrophotometry that 3×10^6 and 3×10^7 dilutions were prepared. Each strain adds to 2 well which one of the wells contains gentamicin but no other. Then plates incubated for 2 h and washing steps finely done by phosphate buffered saline for collapse of Hella cells, were presence of the Triton–X–100 and then the solution of the wells that contain the bacteria moved into brain heart infusion agar. After 24 h incubation in 37 °C, colony count executed. Invasion index was determined by division of obtained bacteria after addition of gentamicin per number of counted bacteria before addition of the gentamicin.

2.5. DNA extraction

Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria.

2.6. MLVA typing

In this study, Five VNTR loci (Lm–10, Lm–11, Lm–32, Lm–23, Lm–TR6) and five related primer pairs that previously designed were used to perform MLVA Technique (Table 1)^[18]. Specificity of all primers was confirmed by the Basic Local Alignment Search Tool.

For amplification of five loci that described above, PCR technique was used. The reaction mix involved of 1 μ L of extracting DNA, 2.5 μ L of 10 \times PCR buffer, 0.5 μ L dNTP (10 mmol/L), 1.5 μ L MgCl₂ (50 mmol/L), 0.4 μ L of Taq DNA polymerase (5 unit/ μ L), 1.25 μ L of each primer and deionized water to a final volume of 25 μ L. Cycling parameters for the PCR protocol were as follow: denaturation at 94 °C for 5 min, followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 22 s, extension at 72 °C for 40 s and final extension at 72 °C for 5 min. These conditions were established using the thermo cycler (Eppendorf, Germany). Electrophoresis of PCR products was performed in 3% agarose gel for 100 min in Tris–acetate buffer and visualized by ethidium bromide staining, then illuminated by UV–trans illuminator and logged by a gel documentation apparatus (UVP Gel Seq Software, England). A 50 bp plus DNA ladder (fermentas) was used as a size reference.

2.7. Compute the number of tandem repeats

The following formula was used to compute the number of tandem repeats (TRS):

$$\frac{\text{PPS} - \text{SLFR} + \text{SRFR}}{\text{Tandem repeat size}}$$

Where PPS refers to PCR product size; SLFR means the size of the left flank region; SRFR is the size of the right flank region.

The flanking regions size previously described^[13,14]. Also,

Table 1Characteristics of selected TR loci for MLVA sub-typing of *L. monocytogenes*.

VNTR locus	Unit length (bp)	Allele (range copy)	Offset (bp)	Repeat	Primers
Lm-10	12	3-9	315	GAAGAACCAAAA	F-CAGATATCGATACGATTGAC R-CAGTTAGTATTTCCAACGTC
Lm-11	12	1-6	103	TTGCTTGTITTTTG	F-GAATAAAATGCTAGATGTGG R-CCGATTCAAAAATAGTAAAC
Lm-23	6	15-42	164	CATCGG	F-TATTTACGGAAAAGACGTAG R-CGTAACCTGCTACCATTAG
Lm-32	6	13-21	84	AACACC	F-AAAGCTTTGCCAGTCAAGT R-TTGTGACTTGGCACTTCTGG
LM-TR6	12	3	232	CCAGACCCAACA	F-AAAAGCAGCGCCACTAACC R-TAAAAATCCAATAACACTCTCGA

to calculate the molecular weight of PCR product from the images gained on the gel, gene tools software (Version 3.08) were used (Figure 1). After analysis and obtaining the molecular weight of PCR products, the number of TRS was calculated using the formula described above (Table 2) and isolates was typed. This means that strains that have 80% similarity or more were placed at one type and other strains placed on the other types.

Table 2Types number, sources and the number of tandem repeats per lux of *L. monocytogenes* isolates.

Strain	Source	LM10	LM11	LM23	LM32	LM-TR 6	Type number
1	Vaginal swabs	3	4	28	19	0	1
2	Rectal swabs	3	4	28	24	0	1
3	Urine	4	0	28	21	0	4
4	Rectal swabs	4	3	28	24	3	5
5	Vaginal swabs	5	3	30	15	3	2
6	Vaginal swabs	5	3	30	15	3	2
7	Placenta	5	3	30	15	3	2
8	Placenta	5	3	31	15	3	2
9	Placenta	5	3	0	15	0	6
10	Vaginal swabs	4	3	31	15	3	3
11	Vaginal swabs	4	3	31	21	3	3
12	Rectal swabs	0	4	38	16	0	7
13	Urine	4	3	31	16	3	3
14	Placenta	4	3	31	16	3	3

2.8. Drawing of phylogenetic tree

For this purpose, the number of tandem repeat of each locus converted to the corresponding sequences of isolates and the evolutionary tree of strains was drawn by MEGA software (Version 6.06).

Table 4Susceptibility of *L. monocytogenes* isolates to 8 antimicrobial agents.

Antibiotic	No. (%) samples														
	Placenta (n=4)			Urine (n=2)			Vaginal swabs (n=5)			Rectal swabs (n=3)			Total (n=14)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Chloramphenicol	1 (25)	1 (25)	2 (50)	0 (0)	0 (0)	2 (100)	2 (40)	1 (20)	2 (40)	1 (33.33)	1 (33.33)	1 (33.33)	4 (28.57)	3 (21.42)	7 (50.00)
Penicillin G	2 (50)	2 (50)	0 (0)	2 (100)	0 (0)	0 (0)	3 (60)	1 (20)	1 (20)	1 (33.33)	1 (33.33)	1 (33.33)	8 (57.14)	4 (28.57)	2 (14.28)
Streptomycin	0 (0)	1 (25)	3 (75)	0 (0)	0 (0)	2 (100)	0 (0)	1 (20)	4 (80)	0 (0.00)	0 (0.00)	3 (100.00)	0 (0.00)	2 (14.28)	12 (85.71)
Tetracycline	1 (25)	0 (0)	3 (75)	0 (0)	1 (50)	1 (50)	1 (20)	1 (20)	3 (60)	0 (0.00)	1 (33.33)	2 (66.66)	2 (14.28)	3 (21.42)	9 (64.28)
Trimethoprim	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	5 (100)	0 (0.00)	0 (0.00)	3 (100.00)	0 (0.00)	0 (0.00)	14 (100.00)
Ciprofloxacin	0 (0)	1 (25)	3 (75)	0 (0)	0 (0)	2 (100)	0 (0)	1 (20)	4 (80)	0 (0.00)	1 (33.33)	2 (66.66)	0 (0.00)	3 (21.42)	11 (78.57)
Ampicillin	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	5 (100)	0 (0.00)	0 (0.00)	3 (100.00)	0 (0.00)	0 (0.00)	14 (100.00)
Erythromycin	0 (0)	1 (25)	3 (75)	0 (0)	0 (0)	2 (100)	2 (40)	1 (20)	2 (40)	1 (33.33)	1 (33.33)	1 (33.33)	3 (21.42)	3 (21.42)	8 (57.14)

No: Number of isolates; R: Resistant; I: Intermediate resistance; S: Susceptible.

3. Result

3.1. Bacterial isolation

In this study, 170 clinical samples were collected during the three years (2010-2013). Clinical specimen obtained from patients with spontaneous abortions hospitalized in Shariati hospital, including: placenta tissue, blood, urine, vaginal and fecal swabs that fourteen strains of *L. monocytogenes* were obtained. Maximum and minimum isolates of bacteria related to vaginal swab and blood sample with 5 and 0 isolates, respectively (Table 3).

Table 3

Resources, the number and percentage of samples examined in this study.

Origin sample	Number (%) of the sample	Number (%) of isolated bacteria
Placenta	53 (31.2%)	4 (7.5%)
Blood	12 (7.0%)	0 (0.0%)
Urine	35 (20.6%)	2 (5.7%)
Vaginal swabs	35 (20.6%)	5 (14.2%)
Rectal swabs	35 (20.6%)	3 (8.5%)
Total	170	14 (8.2%)

3.2. Antimicrobial susceptibility test

The RT5 response of *L. monocytogenes* to 6 the various antimicrobial agents in this study is presented in Table 4. High resistance (57.14) to penicillin was found among isolates. Antibiotic resistance not found for ciprofloxacin, streptomycin, trimethoprim, and ampicillin.

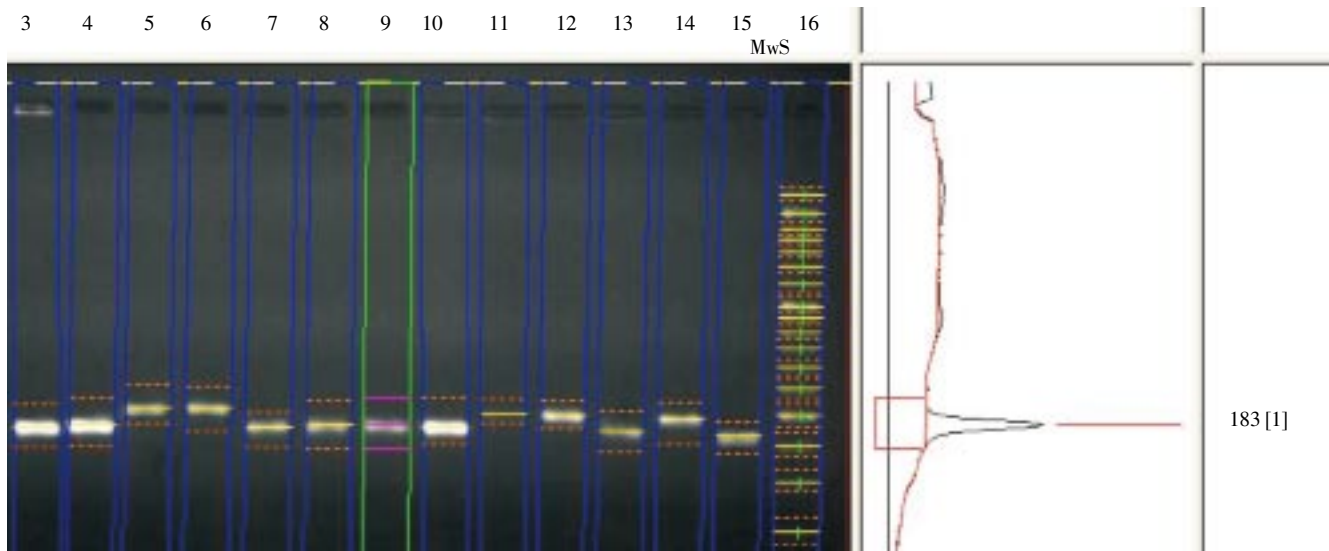


Figure 1. Photograph by gene tools software (Version 3.08) environment for calculation of the size of PCR product.

3.3. Invasion index determination

4. Discussion

The Results of invasion index are presented in Figure 2.

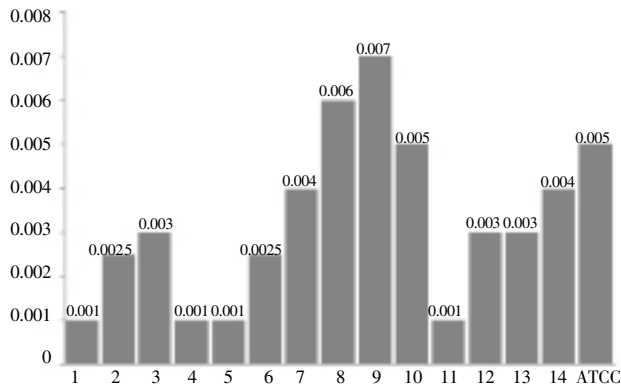


Figure 2. Invasion index of *L. monocytogenes* strains.

3.4. MLVA typing

By using of MLVA technique, fourteen strains studied were located in seven different types that type 2, 3 and 4 strains were consist the most types (Table 2)Evolutionary relationships tree of strains presented in Figure 3.

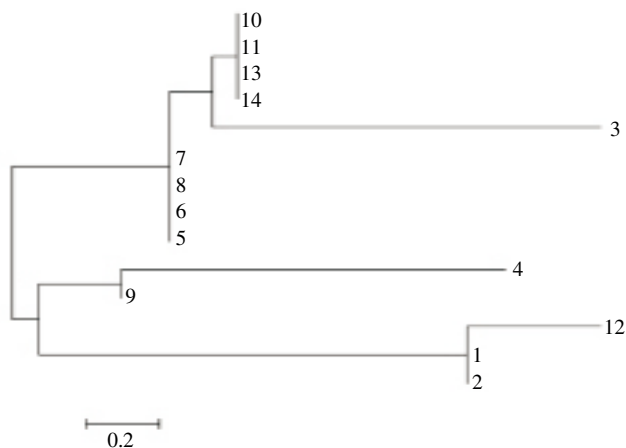


Figure 3. Dendrogram of the MLVA results for the *L. monocytogenes* isolates. The dendrogram is constructed by MEGA 6.06 software.

The incidence of listeriosis in pregnancy is 12 per 100 000, compared with a rate of 0.7 per 100 000 in the common people[19]. Almost one-third of reported human listeriosis cases is reported during prenatal period, which can result in spontaneous abortion (CDC, 2005). Reports of listeriosis from humans in Iran are unclear, either because of losing on recognizing the isolate, unsuitable isolation techniques or absence of awareness. In the present study, of 170 samples collected from women with spontaneous abortions, 14 isolates were identified as *L. monocytogenes*. Contamination rate of samples in women with spontaneous abortion was 4 (7.5), 0 (0), 2 (5.7), 5 (14/2) and 3 (8.5) for placental, blood, urine, vaginal and rectal swab, respectively. Diverse incidence rates of *L. monocytogenes* has been described from some countries. In 2007, Kaur *et al.* collected 305 samples from 61 patients with spontaneous abortions that four isolates were identified as *L. monocytogenes*[20]. In the same year (2007) in Belgrade, one *L. monocytogenes* isolate was separated from 958 clinical samples[21]. In a report in 2013, Soni *et al.*, isolated five (1.7%) *L. monocytogenes* out of the 300 clinical samples[22]. The variance reported among the studies can be due to variances in the population under study include culture, race, nutrition, ecological region and also laboratorial diagnosis methods. Since listeriosis is rare, there are no prospective *in vivo* studies on antibiotic regimens and routinely for treatment of patients penicillin plus an aminoglycoside is used. Nevertheless, reports of resistant isolates of *L. monocytogenes* in recent years have caused concern[6,8,22-24]. In the present study, *L. monocytogenes* was highly sensitive to ampicillin (100%) and trimethoprim (100%), sensitive to ciprofloxacin (78.57%), tetracycline (64.28%), streptomycin (85.71%) and norfloxacin (64.28%) but resistant to penicillin G (57.14%).

Sensitivity of totally strains to ampicillin, similarly seen in the study of Barbosa and colleague. In their study resistances to nitrofurantoin ($n=99$), tetracycline ($n=2$), erythromycin ($n=10$), gentamicin ($n=1$) ciprofloxacin ($n=18$), and rifampicin ($n=1$) also were observed[25]. In confirmation with our study about resistance to penicillin, Prazak *et al.* reported one isolate of *L. monocytogenes* that was sensitive to all antibacterial agents except penicillin[26]. In our study there were strains that were resistant to two or even three antibiotics and two drug-resistant strains have been observed that provide evidence of emergence of multi-resistant *L. monocytogenes* strains, pointing to an increase in the potential menace to human health that poses to this pathogen. In addition, in 2010 a study was conducted on clinical samples in France. The emergence of resistance to tetracyclines and fluoroquinolones was observed but not reported any case of resistance to penicillin[8]. Penicillin resistance in our present is lack of resistance to these antibiotics in the above studies, probably due to differences in drug use patterns among the two countries and the indiscriminate use of antibiotics, and also there is sometimes using of antibiotic without a doctor's recommendation in our country. The invasion index was in the range of 0.001–0.007. Strains 8 and 9 have the invasion index over 0.005, Invasion index of these strains were between 0.006 and 0.007, respectively. These strains were obtained from placental tissue. In study of counter in 2006, the invasion and intracellular growth of 18 strains of *L. monocytogenes* were valued. Index invasive strains were mostly between 0.001–0.007. Only one strain isolated from salmon has a high invasion index (between 0.001–0.7) and was concord with the results of the existent study[27]. In study of Roche *et al.*, on strains with low pathogenicity (low virulence), isolates were investigated complete phenotypically and genotypically to be able to cause little aggressive. In this study, invasion index in strains with low virulence was fewer than one-thousandth[28]. After recognizing strains, typing is very essential. This study is the first report of typing of *L. monocytogenes* by the MLVA technique in Iran. In this study, five loci (LM10, LM11, LM32, LM23, LM-TR6) used for MLVA-typing. This technique successfully discriminated each of isolates and 7 different types obtained that type 2 and 3 with 4 strains were the most abundant types. Murphy *et al.*, in 2006, for the first time were able to differentiate forty-five food-borne *L. monocytogenes* strains using the MLVA. They used the six loci and MLVA successfully discriminated among the isolates[13]. Discriminatory power loci used in this study were consistent with the results of the present study. In 2008 Lindstedt *et al.* studied 79 strains of *L. monocytogenes* isolates from Sweden and these isolates were divided in 28 MLVA Profile, successfully[29]. In another study, Chen *et al.* isolated 46 strains of *L. monocytogenes* from food samples and were typing by the MLVA, with results of the present study corresponded[30]. In this study further strains

isolated from the vagina and the placenta were in a similar type[3,4]. It is interesting that the strains isolated from the vagina and the placenta of the same type were also more resistant to penicillin. This findings show that the source of infection could be same and strains that are available to the vagina can also invade the placenta and have high resistance to penicillin and thus can cause abortion. These results highlight the role of *L. monocytogenes* in abortion. Also, results of the antimicrobial resistance of isolates are indicative of emergence of multiresistant *L. monocytogenes* strains, that pointing to an increase in the potential menace to human health posed to this pathogen. Hence, antibiotic susceptibility testing for on time and precise treatment especially in pregnant women are crucial. Given the importance of finding potential sources of contamination, particularly in epidemics, using a rapid and cheap technique with high contrast resolution is essential. MLVA technique is proper for these purposes, and due to its simplicity, it is recommended. It also recommended further studies onto larger samples from variety of sources such as food and animal specimens to be compare using MLVA.

Conflict of interest statement

Authors declare no conflict of interest.

Acknowledgments:

The authors are indebted to staff and members of the department of Medical Microbiology of Iran University of medical sciences for cooperation.

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