Antimicrobial Susceptibility, Serotyping, and Molecular Characterization of Antibiotic Resistance Genes in *Listeria monocytogenes* Isolated from Pregnant Women with a History of Abortion

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Abstract

Background: *Listeria monocytogenes* show high mortality among pregnant women and newborns. This study aimed to detect *L. monocytogenes* in pregnant women with a history of abortion and assess the serotypes, antibiotic susceptibility patterns, and its resistance genes.

Methods: Overall, 400 vaginal swabs were taken from pregnant women with a history of abortion in the past few years in a tertiary care hospital in Tehran, Iran, during 2015-2018. Antibiotics susceptibility to a panel of 10 antibiotics was determined using the standard disk diffusion method and the isolates serotyped by the agglutination method. The antimicrobial-resistant isolates were also screened for the presence of *tet*M, *erm*B and *dfr*D genes by PCR.

Results: Overall, 22 *L. monocytogenes* isolates were identified. High rates of resistance were observed for trimethoprim (50%; n=11), sulphamethoxazole (50%; n=11), tetracycline (45.45%; n=10) and gentamicin (36.36%; n=8). From 22 *L. monocytogenes* isolates, 13 (59.10 %), 5 (22.73%), 3 (13.63%) and 1 (4.54%) belonged to serotypes 4b, 1/2a, 1/2b, and 3c, respectively. The genetic determinant *tet*M was detected in 70% of the tetracycline-resistant isolates. Out of 11 trimethoprim-resistant isolates, 27.27% isolates contained *dfrD*. Moreover, the *ermB* gene was found in 83.33% of the erythromycin-resistant isolates.

Conclusion: Ampicillin and partly penicillin consider to be suitable antimicrobial agents to treat human listeriosis. Moreover, due to resistance against many antibiotics, it is necessary to continue monitoring and managing antimicrobial resistance.

Keywords: *Listeria monocytogenes;* Genes; Pregnant women

Introduction

Listeria monocytogenes is an important pathogen in pregnant women, neonates, elderly and immuno-compromised individuals and its disease is usually an asymptomatic or mild illness for healthy adults

(1, 2). However, the elderly and immunosuppressive individuals may experience a severe disease such as pneumonia, meningitis and meningoencephalitis (3). *L. monocytogenes* causes the highest abortion rates in newborns (20%-30%) in comparison with other bacteria such as vaginosis bacteria (*Mobiluncus* and *Gardnerella*) (4). More than 30% of pregnancy-associated listeriosis cases result in miscarriage, premature labor, stillbirth, spontaneous abortion or neonatal death (3, 5). The history of prior abortion increases the risk of *Listeria* infection in pregnant women. Women with a history of abortion are susceptible to listeriosis more than those without a history of abortion (6, 7). According to the severe morbidity and high mortality rate of listeriosis in newborns, it is essential to ensure the efficiency of antibiotics against *L. monocytogenes* and monitor the emergence of resistant strains (8).

Drug resistance in *L. monocytogenes* is rarely seen (9). However, some surveys have recently described an increased rate of resistance to one or multiple common antibiotics in some strains isolated from clinical and environmental sources (9, 10). Resistance to antibiotics in *L. monocytogenes* results from the acquisition of genes carried by transferable plasmids and conjugative transposons (11). Moreover, some efflux pumps have also been identified in *L. monocytogenes* (12).

Recently, the incidence of antimicrobial-resistant strains has been increasing (13). The misuse and inappropriate consumption of antibiotics result in the appearance of antibiotic resistance, especially multidrug-resistant (MDR) strains (14). Hence, the identification of *L. monocytogenes* prevalence and its antibiotic resistance seems to be essential (14). In 1988, the first clinical MDR strain of *L. monocytogenes* has been detected with resistance to tetracycline, streptomycin, erythromycin and chloramphenicol (15). After that, different drug resistance patterns to one or several antibiotics in environmental, food and clinical cases have also been identified (16).

Generally, the emergence of MDR strains of *L. monocytogenes* is a growing problem in public health. There are limited data about *Listeria* prevalence in Iran and most cases, the bacterium is not detected or misdiagnosed, which can lead to abortion and severe complications. The objective of present study was to detect *L. monocytogenes* in pregnant women with a history of abortion. Moreover, the isolated strains were also surveyed for their serotypes, antibiotic susceptibility patterns, and resistance potential genes.

Materials and Methods

Specimens and Bacterial isolates

In this cross-sectional study, 400 vaginal swabs were taken from pregnant women with a history of abortion in the past few years in a tertiary care hospital in Tehran, Iran from 2015 to 2017. The inclusion criteria included pregnant women with a history of abortion admitted to a tertiary care hospital in Tehran, Iran. Pregnant women without the history of abortion, healthy pregnant women, and women with other cases of bacterial vaginosis and chronic diseases were excluded from the study.

All specimens were cultured into polymyxin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol (PALCAM, Merck, Germany), Oxford agar (Difco, USA) (17) and selective CHROMAgar (CHROMAgar, Paris, France) media (18) and *Listeria monocytogenes* suspected isolates were tested using standard biochemical tests (such as catalase, oxidase, motility, sugar fermentation tests with rhamnose, xylose, mannitol, α-methyl D-mannopyranoside, motility, hemolysis and CAMP tests) (17) and then confirmed by polymerase chain reaction (PCR) method (19). The isolated bacteria were stored at -80 °C in cryovials (MAST Diagnostics, Merseyside, UK).

Serotyping

All isolates were serotyped by the commercially prepared *Listeria* antisera against somatic (O) and flagellar (H) antigens according to the manufacturer's instructions (Denka-Seiken Co. Ltd., Tokyo, Japan) (20).

Antibiotic susceptibility

Twenty-two *L. monocytogenes* strains isolated from clinical samples were examined for antibiotic sensitivity. Four-five colonies of *L. monocytogenes* isolates were incubated for 24 h at 37 °C in brain heart infusion broth (BHI, Merck, Germany), and then transferred to Mueller Hinton Agar with 5% Sheep Blood (Merck, Germany). The plates were incubated at 37 °C for 24 hours. The antimicrobial susceptibility testing for these isolates was conducted using a procedure by Kirby-Bauer method, according to Clinical & Laboratory Standards Institute (CLSI 2016) disk diffusion assay, with commonly used antibiotics in the hospital: penicillin G (P/10 U), ampicillin (AMP/10 µg), trimethoprim (TS/5 µg), sulfa-

methoxazole (ER/25 µg), tetracycline (TE/30 µg), chloramphenicol (C/30 µg), ciprofloxacin (CIP/5 µg), erythromycin (E/15 µg), gentamicin (CN/10 µg), and streptomycin (S/25 µg) (MAST, UK) (21, 22). The reference strains of *L. monocytogenes* ATCC® 19115™ and *Staphylococcus aureus* subsp. *aureus* ATCC® 25923™ (prepared from Pasteur Institute of Iran) were used for quality control for zone diameter determination.

Molecular analysis

All isolates were screened for the *tet*M, *erm*B, and *drf*D genes using the primers previously described (Table 1) (9).

Table 1: Identities and nucleotide sequences of *L. monocytogenes* resistance gene primers

PCR test	Primer name	Sequence (5′→3′)	Target gene	Size of amplicon	Ref
PCR1	*dfrD*-F	5′-AGAGTAATCGGCAAGGATAACG-3′	*dfrD*	199 bp	[6]
	dfrD-R	5′-AATGGGCAATTTCACAATCC-3′			
PCR2	*ermB*-F	5′-GAAAAGGTACTCAACCAAATA-3′	*ermB*	636 bp	[6]
	ermB-R	5′-AGTAACGGTACTTAAATTGTTTAC-3′			
PCR3	*tetM*-F	5′-GTGGACAAAGGTACAACGAG-3′	*tetM*	405 bp	[6]
	tetM-R	5′-CGGTAAAGTTCGTCACACAC-3′			
F: Forward					
R: Reverse					
bp: base pair(s)					

DNA extraction from bacterial isolates was performed using the Qiagen RNA/DNA Kit (Qiagen, USA) in accordance to the manufacturer's instruments and the extracted DNA was stored at -20 °C.

The PCR reaction mixture (25 µL) included 50 ng of DNA, 2.5 µL of 10X PCR buffer, 1µL MgCl₂, 1-µL of dNTPs, 1 µL of each primer (50 pmol/mL) and 1 U of Taq DNA polymerase (Thermo Fisher Scientific). PCR amplification started with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles (denaturation at 95 °C for 30 sec, annealing at primer-specific temperature for 30 sec, and extension at 72 °C for 30 sec) and a final extension for 10 min at 72

°C. All amplicons were electrophoresed into agarose gel (1% agarose in 0.5 X TBE buffer), stained with ethidium bromide and visualized under a UV light transilluminator.

Ethical approval

The study was approved by the Ethics Committee of Tehran University of Medical Sciences, with number of IR.TUMS.SPH.REC.1395.1485.

Results

Out of 400 clinical specimens, 22 samples were found to be positive for *L. monocytogenes*. Ciprofloxacin showed the highest sensitivity reaching

81.82% (n=18) of the tested isolates. However, high rates of resistance were observed for trimethoprim, sulphamethoxazole, tetracycline, and gentamicin, while low rate of resistance was remarked to ciprofloxacin, ampicillin and penicillin G (Table 2).

Table 2: Antimicrobial resistance profiles of *L. monocytogenes* isolated from clinical samples in Iran

Variable	Dose (µg/disc)	Sensitivity of the isolates (%)		
		R	I	S
Ampicillin	10 µg	0	10 (45.45%)	12 (54.55%)
Gentamicin	10 µg	8 (36.36%)	0	14 (63.64%)
Penicillin G	10 U	0	10 (45.45%)	12 (54.55%)
Trimethoprim	5 µg	11 (50%)	7 (31.81%)	4 (18.18%)
Tetracycline	30 µg	10 (45.45%)	0	12 (54.55%)
Ciprofloxacin	5 µg	4 (18.18%)	0	18 (81.82%)
Sulfamethoxazole	25 µg	11 (50%)	7 (31.82%)	4 (18.18%)
Erythromycin	15 µg	6 (27.27%)	4 (18.18%)	12 (54.55%)
Streptomycin	10 µg	6 (27.27%)	4 (18.18%)	12 (54.55%)
Chloramphenicol	30 µg	6 (27.27%)	6 (27.27%)	10 (45.45%)
R: resistant, I: intermediate, S: susceptible.				

Intermediate resistance to ampicillin 10/22 (45.45%), penicillin G 10/22 (45.45%), trimethoprim 7/22 (36.36%), sulfamethoxazole 7/22 (36.36%), erythromycin 4/22 (18.18%), streptomycin 4/22 (18.18%) and chloramphenicol 6/22 (27.27%) was exhibited by some of the isolates. However intermediate resistance to tetracycline, gentamicin, and ciprofloxacin was not observed in any of the isolated strains. The susceptibility profile of erythromycin and streptomycin was the same with 6 (27.27%) of the isolate been resistant while 4 (18.18%) and 12 (54.55%) were intermediately resistant and sensitive, respectively. Of the 22 isolates, 4 (18.18%) were resistant to only one class of antibiotic, while 18 (81.82%) were resistant to two or more classes antibiotics; none of the isolated strains of *L. monocytogenes* was susceptible to the entire antibiotics tested.

All 22 isolates obtained from clinical samples were serotyped. The predominant serotypes were 4b (13, 59.10%), followed by 1/2a (5, 22.73%), 1/2b (3, 13.63%), and 3c (1, 4.54%).

Twenty-two isolates of *L. monocytogenes* from clinical samples were screened for the presence of *tet*M, *erm*B and *drf*D genes (Table 3, Figs.1-3). The genetic determinant *tet*M was detected in 7 (70%) of the tetracycline-resistant isolates. Out of 11 trimethoprim-resistant isolates, three isolates (27.27%) contained *dfr*D. Moreover, the *erm*B gene was found in 5 (83.33%) of the erythromycin-resistant isolates. Two strains harbored both *tet*M and *erm*B genes simultaneously, while both *tet*M and *drf*D genes were identified only in one strain. As well, one strain carried *tet*M, *erm*B, and *drf*D simultaneously, and twelve had none of the genes.

All patients with listeriosis were reported to specialist. Antibiogram results were also reported to the specialist for appropriate treatment. Furthermore, all patients were followed up for disease progression and recovery. Postpartum follow-up was also performed on all pregnant women with listeriosis. Pregnant women with *Listeria* infection were treated with intravenous ampicillin for 14 d, 2 g every 4 h, and gentamicin, 100 mg every 8 h, followed by ampicillin alone for 14 days. Erythromycin has been used as an alternative to penicillin and gentamicin for the treatment of resistant isolates.

Table 3: Detection of *L. monocytogenes* isolates and resistance profiles used in this study

Isolate	Species	Serotype	tetM	ermB	drfD
*Lm*1	*L. monocytogenes*	4b	-	-	-
*Lm*2	*L. monocytogenes*	1/2b	-	-	-
*Lm*3	*L. monocytogenes*	4b	+	-	-
*Lm*4	*L. monocytogenes*	4b	+	+	+
*Lm*5	*L. monocytogenes*	4b	-	-	+
*Lm*6	*L. monocytogenes*	3c	-	-	-
*Lm*7	*L. monocytogenes*	4b	-	-	-
*Lm*8	*L. monocytogenes*	4b	+	+	-
*Lm*9	*L. monocytogenes*	1/2a	-	-	-
*Lm*10	*L. monocytogenes*	1/2a	-	-	-
*Lm*11	*L. monocytogenes*	1/2b	-	+	-
*Lm*12	*L. monocytogenes*	4b	+	-	-
*Lm*13	*L. monocytogenes*	1/2a	-	-	-
*Lm*14	*L. monocytogenes*	4b	-	-	-
*Lm*15	*L. monocytogenes*	4b	-	+	-
*Lm*16	*L. monocytogenes*	4b	+	+	-
*Lm*17	*L. monocytogenes*	4b	-	-	-
*Lm*18	*L. monocytogenes*	1/2a	+	-	+
*Lm*19	*L. monocytogenes*	1/2b	-	-	-
*Lm*20	*L. monocytogenes*	1/2a	-	-	-
*Lm*21	*L. monocytogenes*	4b	-	-	-
*Lm*22	*L. monocytogenes*	4b	+	-	-

+: Positive/Existence of the gene;
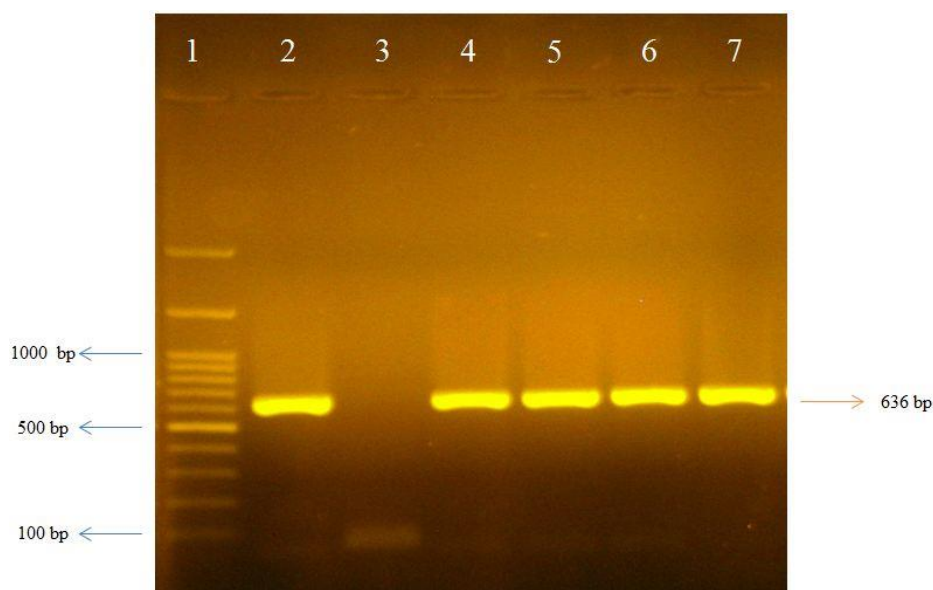-: Negative/Absence of the gene

Fig. 1: Polymerase chain reaction (PCR) analyses of *ermB* gene. Agarose gel electrophoresis of PCR product by using upstream and downstream primers. Amplification of the 636-bp fragment of the *ermB* gene. Lane 1= DNA ladder 100 bp, Lane 2= positive control (*Enterococcus faecalis* ATCC® 29212™), Lane 3= negative control (*Staphylococcus aureus* subsp. *aureus* ATCC® 29213™), Lane 4, 5, 6 and 7= positive results
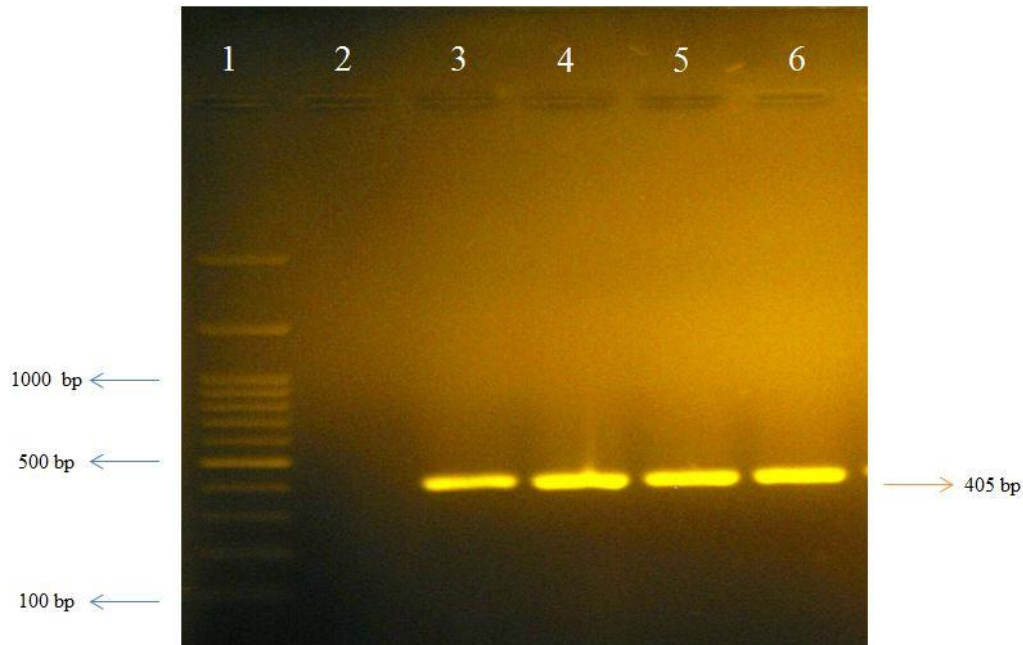
Fig. 2: Polymerase chain reaction (PCR) analyses of *tetM* gene. Agarose gel electrophoresis of PCR product by using upstream and downstream primers. Amplification of the 405-bp fragment of the *tetM* gene. Lane 1= DNA ladder 100 bp, Lane 2= negative control (*Staphylococcus aureus* subsp. *aureus* ATCC® 29213™), Lane 3= positive control (*Enterococcus faecalis* ATCC® 29212™), Lane 4, 5, and 6= positive results
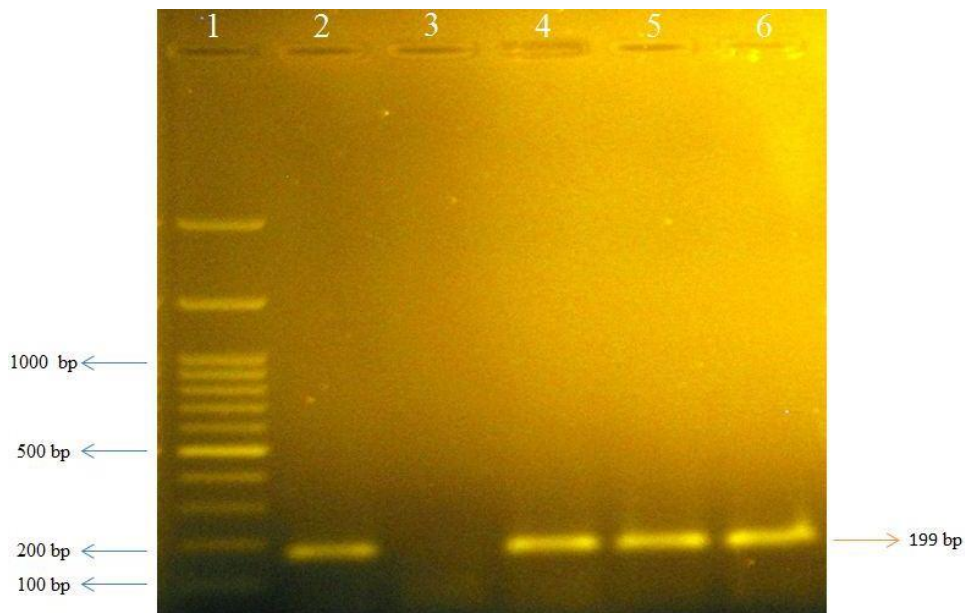

Fig. 3: Polymerase chain reaction (PCR) analyses of *dfrD* gene. Agarose gel electrophoresis of PCR product by using upstream and downstream primers. Amplification of the 199-bp fragment of the *dfrD* gene. Lane 1= DNA ladder 100 bp, Lane 2= positive control (*Enterococcus faecalis* ATCC® 29212™), Lane 3= negative control (*Staphylococcus aureus* subsp. *aureus* ATCC® 29213™), Lane 4, 5, and 6= positive results

Discussion

In the present study, the prevalence of *L. monocytogenes* in pregnant women was 5.5% (22 positives from 400 samples) which is comparable with other studies in Iran. The prevalence of *L. monocytogenes* with human origin was 10% and most of isolates have belonged to 4b, 1/2a, and 1/2b serotypes (23). Similarly, in our study, the predominant serotypes were 4b, 1/2a, 1/2b and 3c.

Antibiotic resistance and inappropriate empirical therapy of listeriosis can lead to increased mortality in patients (24). Although *L. monocytogenes* was distinguished to be susceptible to a wide range of antibiotics during the past two decades, the rate of antimicrobial resistance in *L. monocytogenes* is increasing (13).

In Iran, resistance of *L. monocytogenes* to tetracycline, penicillin G, streptomycin, sulfamethoxazole, gentamicin, erythromycin, and ciprofloxacin has been described (25, 26). MDR *L. monocytogenes* was isolated from veterinary, food, environmental, and clinical samples (27-29). Most of the isolates were resistant to tetracycline, trimethoprim, and sulfamethoxazole, while were sensitive to penicillin G and ampicillin. However, in contrast to our study, most of the strains were sensitive to trimethoprim, sulfamethoxazole, and tetracycline, but were resistant to ampicillin, cefotaxime, and penicillin (30). All isolates were reported to be sensitive to commonly used antibiotics (31). Similar to our study, high resistance to streptomycin and susceptibility to penicillin G and ampicillin were reported (32, 33). In this study, some *L. monocytogenes* isolates were resistant to the most commonly used antibiotics for treating *Listeria* infections including ampicillin, penicillin G, trimethoprim, sulfamethoxazole, erythromycin, streptomycin and chloramphenicol, and these results concur with those found in ducks isolates (34). Conversely, no incidence of resistance to ampicillin, amoxicillin/clavulanate, cefotaxime, meropenem, sulfamethoxazole/trimethoprim, tetracycline, erythromycin, streptomycin, and gentamicin was detected (24).

Penicillin or ampicillin in combination with an aminoglycoside such as gentamicin is considered to be choice therapy for listeriosis (35). However, Co-trimoxazole (trimethoprim/sulfamethoxazole) appears to be a reasonable alternative to ampicillin for the treatment of *Listeria* infection in patients who are allergic to or intolerant of this antibiotic (29, 35).

The genetic determinant *dfr*D was detected in 27.27% of the trimethoprim-resistant isolates. The first clinical strain of *L. monocytogenes* with high-level resistance to trimethoprim was identified in 1995 in France and carried the *dfr*D gene (36). The *dfr*D gene encodes dihydrofolate reductase (DHFR) which is a key enzyme in the tetrahydrofolate pathway, in which it reduces dihydrofolic acid (DHF) to tetrahydrofolic acid (FH4) (37). The commonest mechanism of acquired resistance to trimethoprim (TMP) is a plasmid-mediated production of an insusceptible form of DHFR (37).

In recent years, the increase in the rate of resistance to erythromycin in *L. monocytogenes* is of clinical concern, since erythromycin has been effective for treating pregnant women with listeriosis (38). Among the several mechanisms of erythromycin resistance found in *Listeria*, the most commonly found in strains are methylation of 23S rRNA by *erm* genes (erythromycin ribosome methylase), and drug efflux pumps mediated by the *mef*(A) or *msr*(A) genes (37). In our study, the *erm*B gene was found in 83.33% of the erythromycin-resistant isolates. Some antimicrobial resistance genes were assessed and detected no *erm*B gene in any of the *L. monocytogenes* isolates (13). Instead, *ermA* and *ermC* resistance genes were determined in *Listeria* species respectively (39, 40).

Two types of resistance mechanisms to tetracyclines have been observed in *Listeria* spp.: tetracycline efflux which confers resistance to tetracycline [*tet(*L*)*] and ribosomal protection which confers resistance to both tetracycline/minocycline [*tet(*M*)* and *tet(*S*)*] (37). We found the *tetM* in 70%

of the tetracycline-resistant isolates. The prevalence rate of the *tetM* gene in our study is in accordance with previous studies, in which a high rate of these resistance genes in *L. monocytogenes* isolates was described (13, 29, 41). Two isolates carried *tetM* and *ermB* genes and antimicrobial multi-resistance profiles were found in 10% of isolates (38).

Similar to other previous studies, the current study indicates that the presence of antibiotic resistance genes in *Listeria* isolates did not always associate with phenotypic antimicrobial resistance. Hence, other mechanisms such as reduced permeability, efflux pump activation, mutations, or genes encoding enzymes with antibacterial activities may have attributed to the antibiotic resistance phenotypes (13, 38).

Nugent's criteria are frequently used to identify bacteria via Gram stain of vaginal swabs. The Nugent Score has been used to evaluate 3 types of bacteria via Gram stain including *Lactobacillus* morphotypes, *Gardnerella vaginalis* morphotypes and *Mobiluncus* spp. morphotypes, and do not apply to *Listeria* (42). Therefore, we did not use this method. In this study, the predominant clinical isolates were serotype 4b (59.10%), related to a higher mortality rate in patients infected, especially in infants. These results concur with prior studies. About 60% of the clinical isolates in Brazil were serotype 4b and 19% were serotype 1/2b (43). Serotype 4b was most commonly isolated from clinical samples (44, 45).

The recovery of twenty-two *L. monocytogenes* isolates from cases of pregnant women's vaginal swabs indicates a serious threat to neonates. Most isolates of *L. monocytogenes* had resistance to one or more antibiotics. Although *L. monocytogenes* was distinguished to be nearly susceptible to a wide spectrum of antibiotics in the past few decades, several more recent surveys (13) and results of the present study indicate that the rate of resistance to the different antibiotic in *L. monocytogenes* is growing.

Some challenges were encountered during carrying out some of the purposes of the current study, especially in the course of sampling from some people. A limitation of our study was the fact that sexual practices are part of the individual's private life. Not all of the women in this study felt convenient answering honestly and morally, even when guaranteed secrecy. Matched controls were not included in the study to draw out a conclusion.

Conclusion

Ampicillin and partly penicillin consider being suitable antimicrobial agents to treat human listeriosis. Although the resistance to antibiotics commonly used in human listeriosis treatment is an important public health concern, therefore we recommend regular surveillance of *L. monocytogenes* infections, monitoring antimicrobial susceptibility patterns and rational use of appropriate medications for the treatment of patients.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interests.

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