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Original Research Paper

Evaluation of antibiotic resistance and virulence genes of Listeria monocytogenes isolated from aborted women aborted in Hillah city

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Abstract: Pregnant women who contract *Listeria monocytogenes* may experience invasive disease, miscarriage, stillbirth, and severe infections in the developing fetus. This study sought to identify antibiotic susceptibility patterns, *Listeria monocytogenes* in aborted women, and the genes that encode its virulence factors. **Methods**: In total, 100 specimens were obtained from multiple hospitals in Hilla city between August 2024 and December 2024. Of these, 70 high vaginal swabs were taken from pregnant women with a history of abortion and 17 placental tissue specimens (i.e., pieces of the placenta) were taken from aborted women. Using the conventional disk diffusion method, antibiotic susceptibility to a panel of ten antibiotics was ascertained. actA, inlA, and prfA genes were also checked for by PCR in order to determine the presence of these virulence factors.**Results**: There was a total of 23 isolates of *Listeria monocytogenes* found (14 placenta and 9 vaginal swabs). antibiotic susceptibility teste for streptomycin (96.65%; n = 22), erythromycin (86.95%; n = 20), gentamicin (73.91%; n = 17), and tetracycline (60.86%; n = 14), high rates of resistance were noted. The actA (100 %; n = 23), inlA (86.95 %; n = 20), and prfA (95.65%; n = 22) are the distribution of the *L. monocytogenes* virulence genes. **Conclusion**: Control measures include raising public awareness of listeriosis infections, employing quick diagnostic techniques for efficient treatment, conducting susceptibility testing, choosing the appropriate antibiotics, and preventing the bacteria's effects on expectant mothers.

Keywords: virulence factor genes, PCR, antibiotic susceptibility, *Listeria monocytogenes.*

1. Introduction

Listeria monocytogenes is a Gram-positive, aerobic, intracellular pathogen that tolerates anaerobic conditions. Listeria monocytogenes is most commonly transmitted to humans by the oral route via food, accounting for 99% of cases. Listeria can contaminate raw or ready-to-eat foods such as meat, vegetables, milk, and dairy products. Listeria can grow in the refrigerator for extended periods and can withstand high temperatures (-0.4 to 45°C). Serious consequences of Listeria infection include gastrointestinal inflammation, sepsis, and meningitis in immunocompromised individuals. In pregnant women, Listeria infection is often asymptomatic or influenza-like, making diagnosis difficult and delayed, leading to adverse outcomes for the fetus, as the bacteria can cross the placenta and lead to miscarriage, premature birth, and stillbirth [1]. Given the seriousness of listeriosis and its high infant mortality rate, it is essential to ensure the effectiveness of antibiotics against Listeria monocytogenes and monitor the emergence of resistant strains. Although drug resistance is rare in L. monocytogenes, some strains isolated from clinical and environmental sources have been found to have higher rates of resistance to several common antibiotics, according to recent surveys. The acquisition of transposable genes via conjugative transposons and transferable plasmids is primarily responsible for

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resistance. Furthermore, efflux pumps have been discovered in L. monocytogenes. Antibiotic-resistant strains, especially multidrug-resistant strains, have become more common in recent years as a result of improper use and overconsumption. It is essential that the public health sector in each country prioritize continuous monitoring of antimicrobial resistance and genetic diversity of Listeria monocytogenes isolates in diverse food matrices, given the risk of containing the spread of multidrug-resistant foodborne pathogens.[2] Invasive strains of L. monocytogenes cause severe illness and multiple deaths in humans and animals. A set of virulence genes enhances the pathogenicity of L. monocytogenes. Surface proteins called internalins, encoded by InlA and InlB, mediate bacterial attachment and entry into host cells. Iap is an invasion-associated protein, and actA is another surface protein that regulates bacterial motility in the cytoplasm via actin polymerization. actA, hylA, and inlA are virulence genes thought to be regulated by the prfA gene [3]. Another virulence gene, hemolysin, encodes two virulence factors called listerolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC), which degrade vacuoles by hydrolyzing glycophosphatidylinositol anchors and pore-forming activity. This process allows organisms to escape from the vacuole present in primary phagocytic cells [4]. The aim of this study was to determine the presence of L. monocytogenes in samples from women who had aborted their pregnancy in Hillah, Iraq, evaluate antibiotic resistance in the isolated bacteria, and detect different virulence factors in the isolated strains, which will play a significant role in reducing the risk posed by this bacterium to pregnant women.

2. Methodology

Samples and Bacterial Isolations

In this cross-sectional study, 100 clinical samples (70 vaginal swabs and 30 placental fragments from women with aborted abortions) were collected from several hospitals in Hillah (Imam Al-Sadiq Hospital, Maternity Hospital, and Al-Damen Hospital) between August 2024 and December 2024. All samples were cultured on selective chromogenic medium (a urinary tract infection chromogenic medium), blood agar, and Listeria agar, incubated at 37°C for 24 hours [5]. Suspected Listeria monocytogenes isolates were tested using standard biochemical tests (such as catalase, oxidase, motility, sugar fermentation with rhamnose, xylose, and mannitol, motility, hemolysis, and Kamp tests) [6]. Bacterial isolates were stored in nutrient broth containing 15% glycerol at 20°C. MacFaddin's description, morphological characteristics, and biochemical tests were used to identify isolates to cells and colonies.

Antibiotic susceptibility

The antibiotic susceptibility sensitivity of thirty *L. monocytogenes* isolates that were obtained from clinical specimens was assessed. Mueller Hinton Agar containing 5% sheep blood was used to cultivate the samples (Merck, Germany). For twenty-four hours, the plates were incubated at 37 °C. According to the Clinical & Laboratory Standards Institute (CLSI 2023), the antimicrobial susceptibility testing for these isolates was carried out using the Kirby-Bauer method and the disk diffusion assay. The antibiotics that were commonly used in the hospital were: penicillin G (10 U), ampicillin (10 µg), trimethoprim (5 µg), sulfamethoxazole (25 µg), tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), and streptomycin (10 µg) (MAST, UK). [7,8].

Detection of virulence genes by PCR
DNA Extraction

For L. *monocytogenes* molecular identification, whole genomic DNA was extracted using the manufacturer's standard kit (SolGentTM 1 kb Plus DNA Ladder, Korea).

PCR primers and Conditions

The PCR cycling thermal program settings were used in this reaction to detect the actA, InlA and *prfA* genes. Table 1 shows the PCR primers made by Macrogen (Korea) that were utilized in this investigation.

Table 1: lists the PCR primers and their settings for this investigation.

Primer		Sequence (5----------›3)	Product (bp)	Condition		Cycle No.	Ref.
				Temp	Tim		

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				˚C	e se c		
Act A	F F	CGCCGC GGAAAT TAAAAA AAGA	839	95 60 72	30	30	9
	R	CATGGGT TTCACTC TCCTTCT AC					
prfA	F	CTGTTGGA GCTCTTCT TGGTGAAG CAATCG	106 0	95 62 72	30	30	9
	R	AGCAACC TCGGTAC CATATAC TAACTC					
inlA	F	ACG AGT AACGGG ACA AAT GC	800	95 55 72	30	30	3
	R	CCC GAC AGT GGT GCT AGA TT					

Preparation of PCR Reaction Mixture

An example of the reaction mixture is given in Table 2. The PCR products were run for 60 minutes at 80 volts on a 1.5% agarose gel and stained with 5 milliliters of GoldView (biosharp India). 1 liter of loading dye and 5 liters of amplification products were added to the gel well. The amplicon of the PCR product was scaled using the 100 bp DNA ladder (solGent100bp plus DNA Ladder Korea).

Table 2: PCR reaction mixture

Materials	Volume
Master Mix	12.5 µl
Target DNA	3 µl
F primer (10 pmol/µl)	1.5 µl
R primer (10 pmol/µl)	1.5 µl
Nuclease free water	4 µl
Stain	2.5 µl
Total volume	25µl

3. Results and discussion

From one hundred clinical samples from aborted women, including placental pieces and vaginal swabs, we obtained 23 (23%) isolates of listeria monocytogenes. Out of these isolates, 9 (39.13%) were isolated from vaginal isolates. 14 (60.86%) from the placental pieces. as in table (3) and table (4).

Table 3: The presence of *L.monocytogenes* in aborted women

Positive samples	**Aborted women (n = 100)**					
L.monocytogenes	**Positive Women**		**Sources of samples**			
			Placenta pieces		Vaginal swabs	
	NO.	%	NO.	%	NO.	%
	23	23 %	14	60.86 %	9	39.13 %

Table 4: traits and variables associated with women who were aborted and had an *L. monocytogenes* infection

Age	**Aborted women (n=100)**			
	Examined women		Women with *L.monocytogenes*	
	NO.	%	NO.	%
18 – 25	30	30 %	9	39.13 %
26 – 30	38	38 %	7	30.34 %
31 – 35	18	18 %	4	17.39 %
> 35	14	14 %	3	13.04 %

Biochemical testing and culture techniques were used to identify these isolates. In modified (M1417) listeria agar selective media, L. monocytoge exhibited a small, circular, beta-hemolytic appearance on blood agar, along with a bluish-green color and a yellow hallow surrounding the colonies. Listeria species do not grow in selective CHROMagar (Chromogenic UTI Medium) media. The results of biochemical tests include hemolysis, beta, oxidase, motility, sugar fermentation tests with rhamnose, xylose, and mannitol, and CAMP tests +.

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Antibiotic resistance

95.56 % (n=22) and 86.95% (n=20) of the 23 tested isolates exhibited the highest sensitivity to Penicillin G and Ampicillin respectively. However, (Table 5) shows that high resistance to gentamicin, trimethoprim, chloramphenicol, and ciprofloxacin was low, while resistance to streptomycin, erythromycin, and tetracycline was high.

Table 5: profiles of *L. monocytogenes's* antimicrobial resistance found in specimens

antibiotic	***Dose (µg/disc)***	***Sensitivity of the isolates (%) (n=30)***	
		R	**S**
Ampicillin	10 mg	3 (13.04%)	20 (86.95 %)
Gentamicin	10 mg	17 (73.91%)	6 (26.86%)
Penicillin G	10 U	1 (4.34 %)	22 (95.65 %)
Trimethoprim	5 mg	8 (34.78 %)	15 (65.21 %)
Tetracycline	30 mg	14 (60.86 %)	9 (39.13 %)
Ciprofloxacin	5 mg	7 (30.43 %)	16 (69.65 %)
Sulfamethoxazole	25 mg	9 (39.13 %)	14 (60.86 %)
Erythromycin	15 mg	20 (86.95 %)	3 (13.43 %)
Streptomycin	10 mg	22 (95.65 %)	1 (4.43)
Chloramphenicol	30 mg	4 (17.39%)	19 (82.6 %)

Results of the PCR Assay

The PCR assay was done to identification of the *L. monocytogenes* virulence factors (actA, *inlA*, and *prfA)* is shown in Table 6.

Table 6: Distribution of *L. monocytogenes* virulence factors (actA, *inlA*, and *prfA*).

Gene	**Isolate. No**	**%**
actA	**23**	**100**
inlA	**20**	**95.65%**
prfA	**22**	**86.95%**

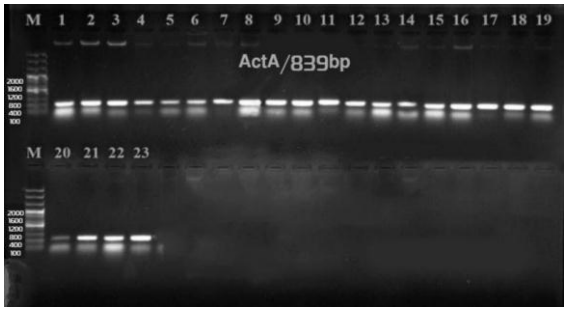

Fig 1: actA PCR product electrophoresis on 1.5% agarose gel using safe red stain at 80 V/Cm took sixty minutes. M: DNA marker ladder.

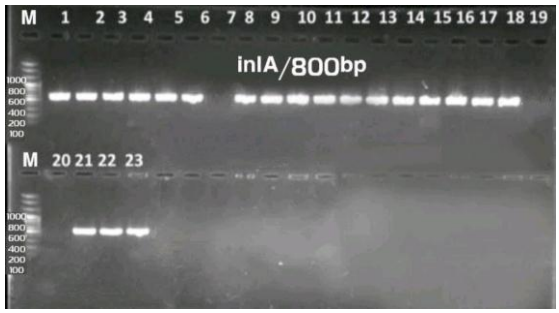

Fig 2: 1.5% Agarose gel electrophoresis of the *inlA* PCR product (800 bp) at 80 V/Cm for sixty minutes using a 1.5% agarose gel and safe red stain. M: DNA marker ladder

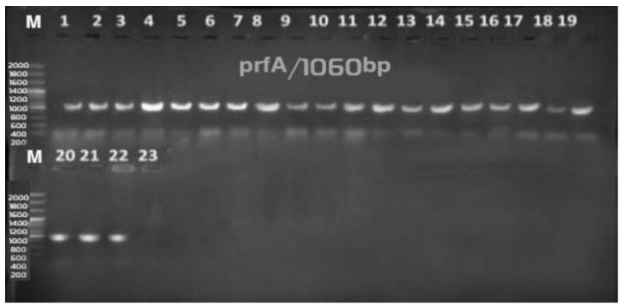

Fig 3: Agarose gel electrophoresis of *prfA* PCR product (1060 bp) ,1.5% agarose gel with safe red stain, at 80 V\Cm, it took sixty minutes. M: Marker DNA ladder

Eating tainted food, such as raw milk, shellfish, and canned meat, is linked to listeriosis infection [10]. Although *L. monocytogenes* can cause a variety of illnesses in elderly, immunocompromised, pregnant, and neonatal patients, it is rare for it to infect the general public [11]. Twenty-three isolates of *listeria monocytogen* were found in clinical specimens used in our study 14 isolates from the placentas of aborted women and 9 isolates from high vaginal swabs of pregnant women with a history of abortion. Al-Mayahi reported different findings, detecting *L. monocytogenes* in 4.8% of women who had abortions [12]. The variance in the findings could be attributed to past medical history, dietary habits, women's immunity, geographical location, contact with contaminated animals, and level of understanding regarding the spread of the disease. Abortion occurs because listeriosis during pregnancy is

difficult to diagnose due to its fever and nonspecific symptoms, particularly in the second trimester [13].

Contact with animals, medical history, and place of residence are risk factors linked to *L. monocytogenes* infection in women. These factors are especially important for women who are pregnant, have had abortions, or have chronic illnesses because they make them more vulnerable to infections due to weakened immune systems. Women who live in rural areas are more vulnerable because they work in agriculture and interact with farm animals and birds. Unhealthy eating practices, such as eating undercooked or unpackaged food, and ignorance of how diseases spread also play a role in the infection's development. Reducing the incidence of infection can be achieved by raising public awareness of the disease's causes, modes of transmission, severity, and preventive measures [3].

Official data on the antimicrobial resistance profile of *L. monocytogenes* in Iraq are lacking [14]. Recent research indicates that *L. monocytogenes* isolated from various sources may be increasingly susceptible to antibiotic resistance. The isolates of *L. monocytogenes* that were found to be resistant to ampicillin, penicillin, and gentamicin, respectively, were 29, (64.4%), 28, (62.2%), and 24 (53.3%) [15,16]. It has been reported that *L. monocytogenes* is resistant to ciprofloxacin, gentamicin, erythromycin, penicillin G, tetracycline, streptomycin, and sulfamethoxazole in Iran [17, 18]. The resistance rate to gentamicin (73.91%), erythromycin (86.91%), and streptomycin (95.65%), as reported in our study, has increased (Table 5).

Gentamicin has long been the first drug of choice for treating listeriosis in humans and animals, while streptomycin and erythromycin are frequently used as a backup option [19]. The results of our study are not consistent with a recent study conducted in Iran (2021), which found resistance rates of 27.27% for streptomycin, 27.27% for erythromycin, and 36.36% for gentamicin [2]. Our results are in contrast to a study by Zena Khalil in Iraq, which reported that *L. monocytogenes* was 100% sensitive to erythromycin [20].

Our study's findings are corroborated by reports of high resistance rates for erythromycin (62.5%) and tetracycline (59.4%) in Egypt and 90.5% to tetracycline and 85.7% to erythromycin in India [21]. Al-Brefkani discovered that among clinical isolates in Iraq, meropenem susceptibility was 85.7%, trimethoprim/sulfamethoxazole (SXT) susceptibility was 57.1%, and ciprofloxacin resistance was intermediate at 50.0% [19]. Nonetheless, our study's results are in line with Yan observation of a low frequency of ciprofloxacin resistance (17.8%) [22]. Clinical isolates in our investigation had a high ciprofloxacin susceptibility (69.65%). Therefore, for patients with listeriosis who are allergic to penicillin, ciprofloxacin can be used as a substitute treatment.

The current study's finding that a sizable percentage of women who had abortions also had high rates of antibiotic resistance is alarming. Continued surveillance is required to identify the resistance profiles of *listeria monocytogenes* in Iraq. The high prevalence of antimicrobial resistance observed in this study is most likely due to the extensive use of these antibiotics in clinical therapy.

A number of factors, such as the infection dosage, the virulence genes in the bacterial strain, and the immunity of the affected individuals, influence an antibiotic's susceptibility *to L. monocytogenes* infection [1]. Individuals with weakened immune systems are particularly susceptible to infections. Upon oral ingestion, *L. monocytogenes* first targets the small intestine mucosa when it enters the human body. From there, the bloodstream and lymph nodes can allow it to spread to other organs. To attack host cells, *L. monocytogenes* uses a variety of proteins, such as internalins that are coded by the inlA and inlB genes. It breaks down the phagocytic vacuole membranes by releasing listeriolysins (*hlyA* gene) and phospholipases (*plcA* and *plcB* genes) as soon as it enters the cell. This allows it to live inside the cell. *L. monocytogenes* reproduces and induces the actA and actB to form actin filaments within the cytoplasm, which helps the bacteria enter the plasma membrane and move between cells to infect nearby cells. Because of this intercellular circulation, *L. monocytogenes* can move between cells and avoid immune responses that are mediated by human T cells. This allows it to infect other tissues and organs, especially the placenta during pregnancy [23].

Using specific primers, PCR was utilized to identify a few virulence genes in *L. monocytogenes* isolates. The *L. monocytogenes* isolates from aborted women carry certain virulence factors, including actA, , *inlA*, and *prfA*. In line with findings published by Parvin who found that L. monocytogenes isolated from a variety of sources,

including blood, urine, feces, placenta, rectum, and vagina samples, as well as livestock and food samples, possessed the virulence factors inlA, inlB, *plcA*, *plcB*, actA, and *hlyA* [24]. actA, *hlyA*, *plcA*, and *prfA* genes encoding virulence factors were found to be present in all *L. monocytogenes* strains that were obtained from patients who had spontaneous abortions Al-mayahi and Jaber, [12].

The virulence factors (actA, *inlA* and *prfA)* that were used in our study with the PCR are 23(100%), 20(86.95%) and 22(95.65%) respectively.

Martínez efound *prfA* genes were present in only 55.6% of the isolates. These gene are the main sources of virulence in *L. monocytogenes* because they are directly linked to the pathogenicity of the bacteria and to the regulation of the expression of other virulence genes in it [25]. The proportion of actA (100%) and *inlA* (86.95%) genes in present study demonstrates the ability of the bacteria to cross the intestinal barrier and to move and spread from cell to cell, invade cells, and cause intracellular parasitism. Consequently, it will be preferable to simultaneously detect virulence genes in a single step as this will save time and labor and be helpful in large-scale surveys to identify virulent strains of Listeria [26].

Our results align with the findings of Arslan and Baytur [27], who discovered virulence factors in all of the *L. monocytogenes* strains they obtained from fresh beef and poultry meat. All of the following genes were detected in the results: hlyA (100%), ActA (100%), inlA (100%), inlB (100%), inlJ (90.2%), prfA (100%), and plcA (100%). These results align with the study conducted by Martínez et al (2023), which reported a high presence of the inlA gene (95.6% of isolates), inlB gene (97.8% of isolates), and ActA gene (100% of isolates) [25].

The major regulator of virulence factors, *prfA*, whose transcription levels were very high 28 (93. 33%) in our study. *prfA* transcription marks the transition from saprophyte to opportunistic style in the intracellular environment. *prfA* transcription is activated during host infection to prevent the transcription of virulence factors when it is not required. Bile salt hydrolase activity is crucial for symptom onset, allowing the overcoming of the obstacles imposed by the gastrointestinal tract. Previous studies reported that mutants with *prfA* deletions are non-virulent due to the inefficient transcription of virulence factors [28]. Listeriosis is associated with an increase in virulence due to the presence of numerous responsible genes, which is consistent with our study's findings. Therefore, determination of infection.

Conclusion

Detection of *L. monocytogenes* and the risk factors associated with infection in aborted women is important for effective treatment and control of listeriosis infection. Detection of virulence genes in the isolated strains is an important indication for its pathogenicity and Infecting pregnant women and causing miscarriage. This study demonstrated the emergence of a significant shift in the incidence of antibiotic resistance in isolates of *L. monocytogenes*, which is to be expected when food manufacturing techniques move from a locally grown food supply paradigm to one that is globally connected. Antibiotic susceptibility testing must be done in advance of antibiotic treatment as a result of this investigation. This would prevent the spread of antibiotic-resistant isolates from these bacteria in Iraqi hospitals and the community, in addition to aiding in the responsible use of antibiotics.

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