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DETECTION OF ANTIMICROBIAL RESISTANCE OF *LISTERIA MONOCYTOGENES* ISOLATED FROM ABORTED COWS IN IRAQ

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ABSTRACT

Listeria spp. is one of the abortion causative agents in animals, especially in ruminants. This work aimed to detect *Listeria* spp. in milk and aborted fetus cows in Iraq. A total of 50 organ samples from aborted cow fetuses, including (brain, liver, and spleen), and 50 milk samples from the same aborted cows were collected from Baghdad farms, Iraq from (October 2023-March 2024). The bacteria were identified by conventional culture methods, biochemical tests, and the VITEK2 compact system, followed by molecular confirmation. The antimicrobial resistance pattern assay was performed using the disc diffusion method against eight antibiotic agents, and the *L.monocytogenes* virulence genes involving *prfA,actA*, and *hyl*A genes were detected using the PCR. The results revealed that only *L. monocytogenes* was detected at 2/50(4%) from aborted fetuses isolated from the brain and liver, while not in milk samples. The *L.monocytogenes* showed 100% resistance against erythromycin, ampicillin, cotrimoxazole, chloramphenicol, vancomycin, and tetracycline. At the same time, all the isolates had a high MDR and MAR (Multiple Antibiotic Resistance) index. This study concluded that *L.monocytogenes* is one of the abortion causative agents in cattle in Iraq, and the high antibiotic resistance of *Listeria* leads to economic loss and a possible risk to humans.

Keywords*: L.monocytogenes,* abortion, cow, antimicrobial resistance

INTRODUCTION

The genus of *Listeria* members is Gram-positive, small rod bacteria. Although, *L.ivanovii*, *L.seeligeri,* and *L.innocua* cause disease sporadically or in outbreaks, a zoonotic *L.monocytogenes* is a potential pathogen spp. that can be distributed in environments including water, soil, animal feed, raw and frozen meat causing foodborne listeriosis, both invasive and non-invasive infection. *Listeria* spp. has been investigated in different animals, including mammals, fish, and birds (Paduro *et al*., 2020; Saleh *et al*., 2024; Yassin *et al*., 2021).

Field animals, including cattle and small ruminants, including sheep and goats, are the most animal species susceptible and infected with *Listeria*, and apparently healthy animals can secrete *Listeria* in their feces in the environment and become a natural reservoir of it at farms (Mohammad *et al*., 2024; Bandelj *et al*., 2018; Rodriguez *et al*., 2021). Various clinical manifestations of

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Listeriosis in animals can be seen, including septicemia, abortion, gastroenteritis, mastitis, central nervous system, and eye infection (Papić *et al*., 2019; Matto *et al*., 2024). Abortion due to Listeriosis is mostly identified in cattle, sheep, and goats. It occurs at high rates in the late stage (third) of gestation. It is a seasonal disease that occurs more frequently in cold weather, particularly during winter or early spring, and is attributed to increased feeding of animals on contaminated silage, which serves as the primary source of infection (Abdlla *et al.,* 2015; Whitman *et al*., 2020; Palacios-Gorba *et al*., 2021).

Listeria has several virulence genes linked with the bacterium's ability to survive and spread in host cells to cause Listeriosis in humans and animals. The main *L. monocytogenes* virulence genes associated with its pathogenesis exist on the *Listeria* pathogenicity island (*LIP1*), among these virulence genes are internalins (*inl A, B, C, and J*), actin (*act*A), Listeriolysin O encoded by (*hyl*A), virulence regulator (*perfA*), phospholipase C (*pl-PLC, plcA)* and invasion associated protein (*iap*) (Sereno *et al*., 2019; Gray *et al*., 2021; Ali and Yassein, 2021). *prfA* is a key activator of the *L. monocytogenes* virulence gene. Upon entering a host cell, the bacterium is enclosed within a single primary membrane vacuole, which is lysed by listeriolysin O and phosphatidylinositol-specific phospho-lipase. This allows *Listeria* to escape into the cytosol, where it multiplies and spreads within the cell. The *actA* gene plays a critical role in *L. monocytogenes* intracellular motility, enabling the bacterium to move from cell to cell (Prokop *et al*., 2017; Osman *et al*., 2020).

The emergence of *Listeria* resistance to antibiotics has been a growing concern since it was first reported in 1990 (Poyart-Salmeron, 1990). Several published reports have investigated the antibiotic resistance of *Listeria* spp, especially *L.monocytogenes* against the antibiotics taken as the drug of choice to treat Listeria infections, such as tetracycline, penicillin, ampicillin, cephalosporin 3rd and 4th generation and others in humans and animals (Shourav *et al*., 2020; Babacan, 2021).

MATERIALS AND METHODS

Ethical approval

Ethical approval was granted through the Local Committee of Animal Care and Use at the College of Veterinary Medicine within the University of Baghdad (Number PG1010/16/5/2024).

Samples collection

Fifty cow abortion cases were investigated during the study period, from October 2023 - March 2024. About 10-25 grams of cows' aborted fetus organs (liver, spleen, and brain) and milk samples from the same aborted cow were collected with aseptic conditions and transported to the zoonoses laboratory under cold conditions. The surveillance data included the age of the animal, gestation period, and date of abortion (months).

Identification

The identification of *Listeria* was done according to (ISO, 2017; Procop *et al.,* 2020) with some modifications. Aseptically, the fetus organ sample and 225ml of *Listeria* enrichment broth (Oxoid, UK) were transferred into a sterile stomacher bag and homogenized using a stomacher (Stomacher, Germany). After incubation for 24-48 hrs at 4°c (cold enrichment), weekly, a loopful from enrichment broth was plated onto Blood Agar (Himedia, India), incubated for 24-48hrs, then small pinpoint colonies with or without hemolysis on blood agar were streaked onto *Listeria* Oxford medium(HiMedia, India), then incubated for 24-48hrs at 37°c, black colonies surrounded by blacking media(esculin hydrolysis) were plated on HiCrome *Listeria* agar (HiMedia, India) and incubated for 24-48hrs at 37°c, blue surrounded by halo zone colonies were picked up and subjected for grams stain, catalase test, fermentation of mannitol and rhamnose, motility at 25°c, and CAMP test.

For further confirmation, molecular detection was performed by PCR assay using iap gene: mono 5′-CAAACTGCTAACACAGCTACT-3′, and Lis1B- 5′-TTATACGCGACCGAAGCCA AC- 3′ (Bubert *et al*., 1999). Genomic DNA from the isolated *Listeria* colonies was extracted using FavorPrep Total DNA Mini Kit (FAVORGEN, Korea). The PCR in a final volume of 25µl reactions containing master mix (12.5 µl), forward and reverse primer (1 µl to each), genomic DNA (1.5 µl), and distal water (9 µl) in Applied Biosystems™ ProFlex™ PCR System (Fisher Scientific, USA). The PCR conditions were: initial step at 95°c for 3 min, one cycle; 35 cycles of denaturation at 95°c for 30 sec, for 45 sec at 57°c as annealing step and at 72°c for 1 min as extension1; and finally extension 2 at 72°c for 10 min (1 cycle). After that, the PCR products were fractionated on 1.5% agarose gel electrophoresis. The bands were visualized using a red stain under an ultraviolet illuminator (Vilber Lourmat Sté /France). The PCR amplifications were sent for partial sequencing by Macrogen Corporation, Korea, and the data were analyzed by BLAST at the NCBI.

Virulence genes detection

Listeria isolates were screened for the presence of *prf*A, *hyl*A, and *act*A virulence genes, the sequence of the primers listed in **Table 1**. The PCR in a final volume of 25µl reactions containing master mix (12.5 µl), forward and reverse primer (1 µl to each), genomic DNA (1.5 µl), and distal water(9 µl) in Applied Biosystems™ ProFlex™ PCR System (Fisher Scientific, USA), the amplification condition were initial step: one cycle for 2 min at 95°C; 35 cycles of denaturation for 30 sec at 95°C, annealing for 45 sec at 57°C for *perf*A and 60°C for both *hyl*A and *act*A and extension 1 at 72°C for 1min, and finally, one cycle for extension 2 at 72°C for 7min. After that, the PCR products were fractionated on 1.5 agarose gel electrophoresis. The bands were visualized using a red stain under an ultraviolet illuminator (Vilber Lourmat Sté /France).

Table 1: The primers used in this study

Primer	Sequence (5'--3')	Size (bp)	References
hlyA	F-GCAGTTGCAAGCGCTTGGAGTGAA	456	(Nayak *et al*., 2015)
	R-GCAACGTATCCTCCAGAGTGATCG		
prfA	F-GTATTTTTCTATATGATGGTATCAC AAAGC	540	(Johnson *et al*., 2004)
	R-CATATCTTTTGAGATAATCAAGATT TTGTAC		
actA	F-CGCCGCGGAAATTAAAAAAAG	839.	(Nayak *et al*., 2015)
	R-ACGAAGGAACCGGGCTGCTAG		

Determination of antimicrobial resistance pattern

The following antibiotic agents are gentamicin 10 mg, chloramphenicol 30 mg, cotrimoxazole (sulpha/Trimeth) 25 mg, tetracycline 30 mg, erythromycin 15 mg, ciprofloxacin 5 mg, ampicillin 10 mg (HiMedia, India) and vancomycin 30 mg (Liofilchem, Italy) were used for the antimicrobial resistance of *Listeria* isolates using disc diffusion method based on European Committee on Antimicrobial Susceptibility (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) (EUCAST 2024; CLSI, 2022). Briefly, a bacterial suspension 1x108 cfu/ml was prepared using (McFarland 0.5 standard tube), then, a

cotton swab was sunken in bacterial suspension and spread equally over the Muller-Hinton agar (HiMedia, India), left for 15 minutes, and the discs of antibiotics were placed onto the Muller-Hinton agar as five discs per plate and incubated at 37°C for 24hrs. Then the diameter of inhibition zone was measured and evaluated as Resistance (R), Susceptible (S) or Intermediate (I) based on the CLSI for *Staphylococcus* spp (ATCC 25923) and EUCAST for *L.monocytogenes*, Multi drug resistance (MDR) and multiple antibiotic resistance (MAR) index were done according to (Magiorakos *et al*., 2012; Sandhu *et al*., 2016).

RESULTS

Identification of *Listeria* spp

Two isolates were picked up based on cultural, biochemical, and CAMP tests, which were suspected as *L.monocytogenes*. The isolates appeared as small, gray colonies with ß hemolysis (narrow zone) on sheep blood agar, surrounded by blacking media (esculin hydrolysis) on Oxford medium, and blue colonies with a yellow zone on HiCrome *Listeria* agar, positive CAMP test with ß- hemolysis *Staphylococcus aureus*, catalase test and motile at 25°C. Gram stain reaction the isolates appeared as small rods Gram-positive as Chinese letters (Figure 1).

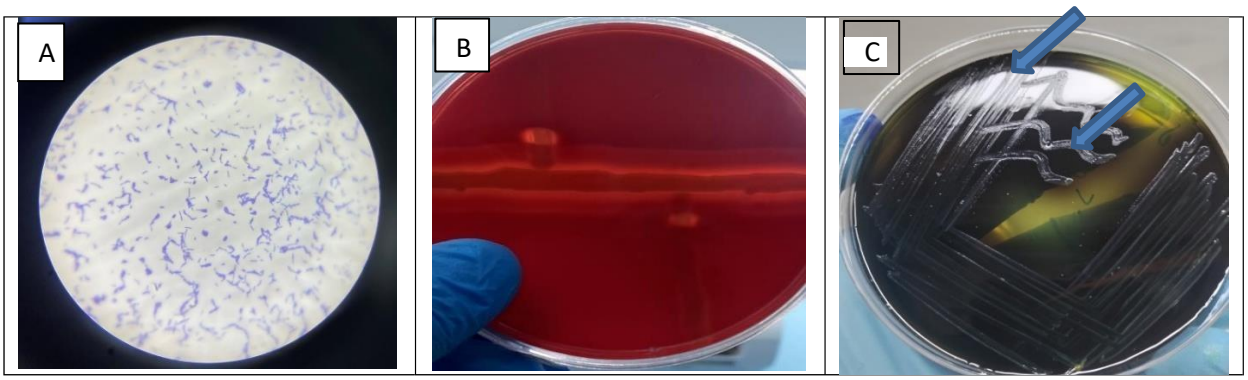
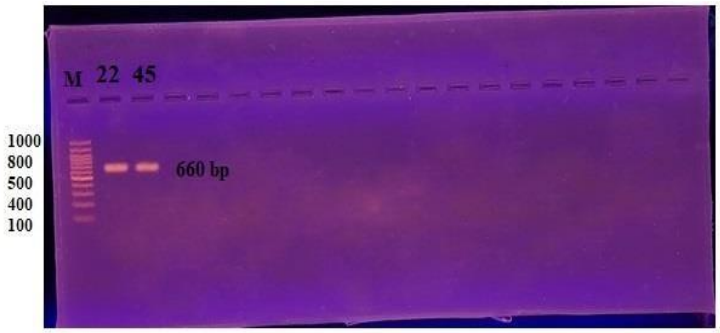


Figure 1: A: Gram stain showed gram-positive small rods, B: on Oxford media, colonies surrounded by black media (esculin hydrolysis) C: CAMP test showed positive with ß- hemolysis *Staphylococcus aureus*

Molecular analysis using the *iap* gene, the two isolates yielded an amplification of 660pb for *L.monocytogenes* (Figure2), and the partial sequences of the isolates had 99% homology with *L.monocytogenes* strain **CP007198.1** and the isolates were deposited under accession numbers PP786247.1 and PP786248.1 in Gen-Bank.



Figur. 2. Agarose gel electrophoresis 1.5% of PCR of *iap* gene (660pb) for *L.monocytogenes* isolates (22 and 45) Lane: M (M: 100bp ladder)

The results showed that the overall isolation rate of *Listeria* from aborted cow fetuses samples was two out of 50 (4%) as *L.monocytogenes*, which isolated from the brain and liver, while not isolated from the milk of the same cows. According to the stage of gestation and age of cows, *L.monocytogenes* was identified in the third stage 2/31(6.4%), and only from 3-6 years old 2/29(6.8%) (from 3 and 4 years) old cows. In addition, *Lmonocytogenes* was reported in December 1/11(9.1%) and January 1/8 (12.25%) (Tables 2,3 and 4).

Table 2: Occurrence of *L.monocytogenes* according to the stage of gestation

Stage of gestation (months)	No. of samples	Positive result	%
First stage (1-3)	0	0	0
Second stage (4-6)	19	0	0
Third stage (7-9)	31	2	6.4
Total	50	2	4

Table 3: Occurrence of *L.monocytogenes* according to age

Age of animals (years)	No.	Positive result	%
3-6	29	2	6.8
7-10	21	0	0
Total	50	2	4

Table 4: Occurrence of *L.monocytogenes* according to months

Month	No. of samples	Positive result	%
October/2023	5	0	0
November/2023	5	0	0
December/2023	11	1	9.1
January/2024	8	1	12.5
February /2024	7	0	0
March/2024	1	0	0
Total	50	2	4

Antimicrobial susceptibility profile

L.monocytogenes isolates were 100% resistant against erythromycin, ampicillin, trimethoprim-sulfamethoxazole, chloram-phenicol, vancomycin, and tetracycline, while susceptible 100% to gentamycin and ciprofloxacin, both the isolates were 100% MDR, and the MAR index was 0.75 (22 (Table 5).

Table 5: MDR profiles, and MAR index for *Listeria* isolates from cows' aborted fetuses.

Listeria spp	No. of antibiotics for 8 antibiotics	MAR Index	MDR profile
L.monocytogenes	6	6/8(0.75)	E AMP COT C VAN TE
L.monocytogenes	6	6/8(0.75)	E AMP COT C VAN TE

E: erythromycin;AMP= ampicillin;COT: trimethoprim-sulfamethoxazole; C: chloramphencol; VAC: vancomycin;TE: tetracycline

Virulence genes determination

*act*A, *hyl*A, and *prf*A virulence genes were detected in both *L.monocytogenes* isolates (Fig. 3).

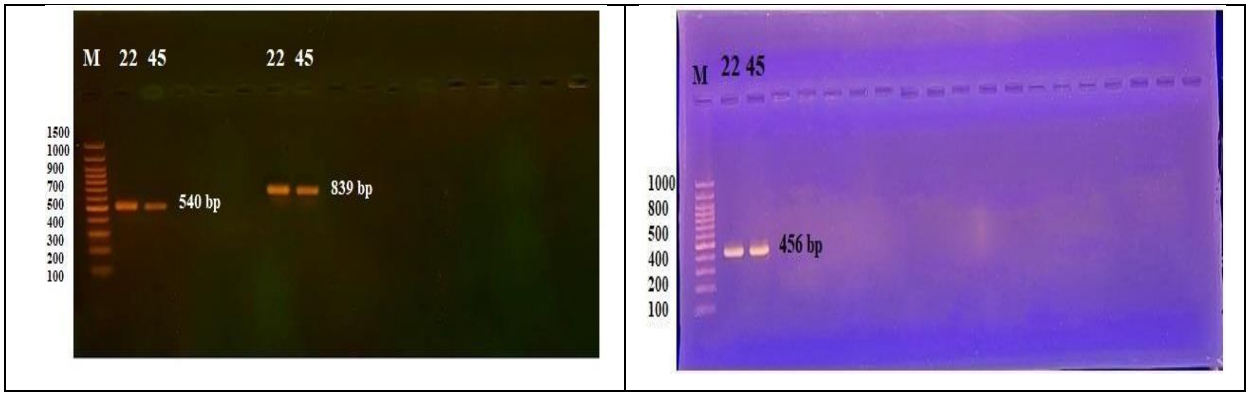


Figure 3: Agarose gel electrophoresis 1.5% of *act*A (839pb), *hyl*A (456pb) and *prf*A (540pb) virulence genes amplification of *L.monocytogenes* isolates (22 and 45). Lane: M (M: 100bp ladder).

DISCUSSION

Abortions caused by Listeria in ruminants have been detected around the world and the rate of incidents has varied from outbreaks to sporadic cases (Whitman *et al*., 2020, Šteingolde *et al*., 2021; Esposito *et al*., 2020; Gradovska *et al*., 2023). Although *Listeria* was isolated in Iraq from humans (Heba *et al*., 2024; Abdalla *et al*., 2004), animals and animal products (AL-Shamary., 2009; AL-Shamary and Najim, 2009; Hamzah *et al*., 2010), there are limited researches available on *Listeria* spp in aborted cows.

In the current work, only *L.monocytogenes* were detected in cow aborted fetuses 4%, and not identified in milk samples collected from the same cows. This result is the first study of *L.monocytogenes* in aborted cows in Baghdad city. This result is not consistent with a study that recorded a high rate of 25% *L.monocytogenes* in aborted cow fetuses and 28.6% in milk samples of the same aborted cows using molecular identification in Salahudeen province (Noomi *et al*., 2021). In Kerbala, among bacteria, *L.monocytogenes* was 0.83% in aborted fetuses and vaginal swabs of cows, which were lower than reported in the present study (Kadim 2013). In Latvia, the prevalence of *L.monocytogenes* was 16.1%, 16.1 and 23.7% (Šteingolde *et al*., 2013; Šteingolde *et al*., 2014; Šteingolde *et al*., 2021). Also, *L.monocytogenes* recovered from two aborted fetuses from an outbreak of cattle abortion (Whitman *et al*., 2020), nearly similar to the current study *L.monocytogenes* recorded at 4.66% in bobbin abortion cases. In Turkey, *Listeria* spp was identified in 5.6% of the vaginal swabs in herds of cows with abortion (Akca and Şahin, 2011).

The occurrence of *L.monocytogenes* rises in flock animals during the cold climate season (Castro *et al*., 2018, Palacios-Gorba *et al*., 2021). In the current work, *L.monocytogenes* were identified in December at 9.1% and high in January at 12.5%, these months are among the winter cold months in Iraq. Coordinated with studies, the incidence of *L.monocytogenes* in aborted cases was higher in spring, 22.3%, 13% in winter and summer, and 13.9% in autumn (Šteingolde *et al*., 2021).

L.monocytogenes isolates were detected only from 3-6 years of age in this study, which agrees with a finding that detected the bacterium more frequently under four years of age at 61%. Another study found *L. monocytogenes* in aborted cattle significantly at 3 years of age (Šteingolde *et al*., 2021; Šteingolde *et al*., 2014).

In the present study, *L. monocytogenes* was observed during the third trimester of gestation. This finding aligns with previous studies that reported a high prevalence of Listeria-associated abortions occurring during the third stage of gestation (Barkallah

et al., 2014; Šteingolde *et al*., 2014; Whitman *et al*., 2020).

The difference in results among studies is attributed to several factors, including changes in management, weather, physiological changes, stress conditions, immune status, geographical area, factors related to samples, such as source, type, and size of samples, and natural genetic differences of the *Listeria* strains (Roberts *et al*., 2003; Mackiw *et al*., 2020; Moabelo *et al*., 2023).

Listeria spp are varied in their pathogenicity. Some strains are virulent and cause infections with high rates of mortality and morbidity, whereas other strains are non-virulent and do not cause diseases. In the current finding, *L.monocytogenes* isolates harbored the *prf*A, *hlyA*, and *actA* virulence genes, which indicated these strains are virulent and pathogenic. These genes were recorded in cow samples, such as aborted fetuses, and raw beef at 87.5%, and in mild beef at 50%, and milk (Owusu-Kwarteng *et al*., 2018; Šteingolde *et al*., 2021; Moabelo *et al*., 2023).

A MAR index is a method used to detect bacterial infections and antimicrobial resistance. A MAR index of ≥ 0.2 indicates that the bacteria likely originate from high-risk, contaminated environments where antibiotics are frequently misused (Sandhu *et al*., 2016). A high MAR index was reported in *L.monocytogenes* isolates (Al-Gburi, 2020; Aksoy *et al*., 2018; Shourav *et al*., 2020). In the current study, *L.monocytogenes* isolates were resistant against erythromycin, ampicillin, trimethoprim-sulfamethoxazole (COT), chloramphenicol, vancomycin, and tetracycline, while were sensitive to gentamycin and ciprofloxacin, with MDR and high MAR index. There are no studies on the antimicrobial resistance of *Listeria* spp isolated from aborted cow cases to compare with the present study. However, *Listeria* isolated from cattle environments with raw milk, water, food, and dung, showed resistance against erythromycin, ampicillin, (91.7%), trimethoprim, and tetracycline (75%), and was susceptible to gentamicin with high MAR index ranging from 0.4-0.64 (Aksoy *et al*., 2018; Shourav *et al*., 2020). The increased resistance patterns to penicillin, amoxicillin, ampicillin, and tetracycline may attributed to overuse in veterinary medicine and the use of antibiotics in food supplements for animals (Caneschi *et al*., 2023; Shourav *et al*., 2020).

CONCLUSION

L.monocytogenes caused abortion at 4% in Baghdad City, and occurred more in December and January months (cold winter months) in Iraq, in the late stage of gestation, and at 3-6 years of age. *L.monocytogenes* were pathogenic strains and highly antimicrobial-resistant and a high MAR index indicated the Listeria strains were exposed to different and numerous antimicrobials.

High light

1. Investigated the occurrence of *Listeria* in aborted cow fetuses and milk of the same aborted cow.
2. Detected the virulence-associated genes.
3. Detection of the antimicrobial resistance, MDR, and MAR index.

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الكشف عن مقاومة المضادات الحيوية لبكتيريا الليستيريا المستوحدة المعزولة من الأبقار المجهضة في العراق

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تعد بكتريا الليستيريا المستوحدة أحد انواع البكتريا المسببه للامراض المعدية في الحيوانات وهو أحد العوامل المسببة للاجهاض في الابقار. أجريت هذه الدراسة للكشف عن بكتريا الليستيريا في أجنه الأبقار المجهضة وكذالك في عينات حليب لنفس الأبقار. تضمنت هذه الدراسه اخذ 50 عينة من الأعضاء الداخلية (الدماغ, الكبد والطحال) لأجنة أبقار مجهضة و50 عينة حليب لنفس الأبقار. جمعت العينات من مدينه بغداد ,العراق للفتره من (تشرين الأول 2023_اذار 2024). تم اجراء العزل بواسطة طرق الزرع البكتيري, وطرق الكيمياء الحيوية واستخدام نظام الفايتك وطرق التحليل الجزيئي. باستخدام طريقة الانتشار القرصي تم الكشف عن مقاومة الليستيريا لثمانية مضادات حيوية, إضافة إلى التحري عن ثلاث جينات عوامل الضراوة. اظهرت النتائج عزل فقط الليستيريا المستوحدة وبنسبة 4% من الأجنة المجهضة بينما لم تعزل من عينات الحليب. كذلك اظهرت النتائج مقاومة الليستيريا المستوحدة للفانكومايسن وارثرومايسين وتيتراسايكلين وترايمثيبروم سلفاميثازول وكلورامفنكول. إضافة إلى ذلك, اظهرت كل العزلات تعدد المقاومة للمضادات الحيوية ومؤشر متعدد المقاومة مرتفع. استخلصت هذه الدراسة أن وجود بكتريا الليستيريا المستوحدة في أجنة الأبقار والتي كانت مقاومة للمضادات الحيوية وتحمل جينات ضراوة تسبب خسائر اقتصادية عالية في الأبقار الحلوب وخطرا محتملا على الصحة العامة .

الكلمات المفتاحيه: الليستيريا المستوحدة ,الاجهاض, أبقار, جينات ضراوة