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Molecular Detection of Ovine Listeric Abortion in Nineveh Governorate, Iraq

Irak'ın Nineveh Vilayetinde Koyun Listerik Abortunun Moleküler Tespiti

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ABSTRACT

Listeriosis is an important abortifacient sheep disease, and is considered one of the most risky bacterial zoonotic disease worldwide**.** The study was carried out in 50 sheep flocks were located in the Nineveh governorate, Iraq during November and December 2022. A total of 300 specimens of blood, abomasal content and brain (100 each) were obtained from local breed ovine aborted fetuses (in the last stage of gestation) to be tested for molecular detection of *Listeria monocytogenes L. monocytogenes* DNA was detected in a total of 61(20.3%) specimens, distributed as: 35(57.4%), 15(24.6% and 11(18.0%) strains from fetal brain, abomasal content and blood specimens respectively using direct genus-specific conventional polymerase chain reaction (prfA gene) C- PCR. Two *L. monocytogenes* strains (HMB1 listeriolysin, HMB 2 listeriolysin) deposited in GenBank under accession numbers LC769365.1, and LC769366.1. Al *L. monocytogenes* strains were positive for three genes (*InlJ, InlA, and* hlyA) except act A gene was detected in 46 (75.4%) strains. In conclusion, *L. monocytogenes* is one of the important causative agent of abortion in sheep flocks in Nineveh governorate, Iraq, and greater fetal brain specimens were positive for listerial infection compared with other specimen.

Keywords: Listerosis, abortion, ovine, listeriolysin, Iraq

ÖZ

Listeriyoz, dünya çapında en riskli bakteriyel zoonotik hastalıklardan biri olarak kabul edilen önemli bir abortif koyun hastalığıdır. Çalışma, Kasım ve Aralık 2022 tarihlerinde Irak'ın Nineveh vilayetinde bulunan 50 koyun sürüsünde gerçekleştirildi. Listeria monocytogenes'in moleküler tespiti için yerel ırk koyun abort fetüslerden (gebeliğin son aşamasında) toplam 300 adet kan, abomazum içeriği ve beyin örneği (her birinden 100 adet) alındı. L. monocytogenes DNA'sı, doğrudan cinse özgü konvansiyonel polimeraz zincir reaksiyonu (prfA geni) C-PCR kullanılarak alınan toplam 61 (%20,3) örnekte tespit edildi. Örneklerin dağılımı: fetal beyin örneklerinden 35 (%57,4) suş, abomazum içeriği örneklerinden 15 (%24,6) suş ve kan örneklerinden 11 (%18,0) suş şeklindeydi. İki L. monocytogenes suşu (HMB1 listeriyolizin, HMB 2 listeriyolizin) GenBank'a LC769365.1 ve LC769366.1 erişim numaraları altında kaydedildi. Tüm L. monocytogenes suşları, akt A geni hariç olmak üzere üç gen (InlJ, InlA ve hlyA) için pozitif bulundu. Akt A geni 46 (%75,4) suşta tespit edildi. Sonuç olarak, L. monocytogenes, Irak'ın Nineveh vilayetindeki koyun sürülerinde yavru atmanın önemli nedenlerinden biridir ve diğer örneklerle karşılaştırıldığında daha fazla fetal beyin örneği listeriyal enfeksiyon açısından pozitif bulunmuştur.

Anahtar Kelimeler: Listeriyoz, abort, koyun, listeriyolizin, Irak

INTRODUCTION

Globally, ovine abortion is a common clinical issue and is mostly caused by *L. monocytogenes*.[1] It is a gram-positive, facultative intracellular microorganism that invades and colonizes mammalian cells. It is responsible for three main aspects in ruminants such as abortion in the last trimester of gestation, either sporadically or as outbreak, septicemia and encephalitis.[2] Silage, hay, bedding, and water were considered as major sources and possible reservoirs of *L. monocytogenes* in the farming.[2,3] A third trimester listeric infection may cause fetal death and placental retention with minor maternal sequelae, while near term infection potentially causes serious complications for pregnant dams including dystocia, severe metritis, and septicemia.[2,4]

The pathogenesis of *L. monocytogenes* is boosted by a numerator of essential virulence factors, counting endotoxin (encoded by inlA and inlB), hemolysin (hlyA), phosphatidylinositol-specific phospholipase C (PI-PLC, plcA), phosphatidylcholine-specific phospholipase C (PC-PLC, plcB) and actin polymerizing protein (actA)[5].

Isolation of the bacteria provides a definite diagnosis of the infection, but it takes a elongated time to cultivate on the agar media, L. monocytogenes can be recovered from an agar plate that has been refrigerated or preserved at room temperature for up to 3 weeks, hence it is preferred to use virulence genes to identify it.[6] Molecular techniques have been considered superior to traditional diagnostic methods, especially in recent years where it was relied upon in the diagnosis and classification of bacteria and the identification of virulence factors.[7,8] In Iraq, ovine Listeric abortion is inadequately investigated. Prior studies have been restricted to the bacterial isolation and molecular detection of microorganisms from foodstuffs and aborted cows.[9,10]

The purpose of present study to molecular diagnosis of ovine listeric abotion in Nineveh governorate, Iraq.

MATERIALS AND METHODS

Sample Collection

During November - December 2022, one hundred aborted fetuses from 50 flocks in the Iraqi Nineveh governarate were screened for the presence of *L. monocytogenes*. A 300 specimens were collected from the blood, abomasal content, and brain of the ovine aborted fetus in the last stage of gestation. Each obtained materials were collected separately in a sterile plastic bag and quickly transferred to the laboratory in cooled condition.

Conventional Polymerase Chain Reaction

A 25 mg of foetal tissue specimens were homogenized in phosphate buffered saline (pH 7.4) using mortar and pestle. DNA extraction was executed from the tissue homogenates using the commercial DNeasy Blood &Tissue Kit (Presto™ Mini gDNA Bacteria Kit, Geneaid Biotech Ltd /Taiwan) according to the manufacturer's instructions. DNA extraction were stored at -85 °C until required for PCR analyses. DNA from *L. monocytogenes* ATCC-7644 was used as a positive control, and a DNase-free distilled water was used as a negative control.

For the detection of the genus *Listeria, prs* gene amplification assays were carried out by PCR, using the luniversa l primers UNI-F and UNI-R (5'-'TTAGTGGCGGACGGGTGA and -3' GGTATCTAATCCTGTTTGCTC that amplify a 700-bp fragment of the 16s rRNA gene,[8] and for *Listeria monocytogenes*, the primers *prfA*-R-*prfA*-F (5'- GATACAGAAACATCGGTTGGC and 3' GTGTAATCTTGATGCCATCAG -) that generate a 274-bp fragment of the *prf A* gene.[11] The thermocyclar program were mentioned in table 1. The thermocyclar program for the *prfA* primers (primers *L. monocytogenes)* were presented in table 2.[12]

Table 1: PCR thermocycler program for *Listeria* spp.

The steps	Temp °C	Period	No of cycles
Initial denaturation	95	5 Min.	1
Denaturation	95	45 Sec.	
Annealing	63	45 Sec.	30
Extensions	72	45 Sec.	
Final extensions	72	7 Min.	1

Temp: temperatures, Min: minutes, Sec: seconds

Table 2: The thermocyclar program for the *prfA* primers

The steps	Temp. °C	Period	No. of cycles
Initial denaturation	95	5 Min.	
Denaturation	94	45 Sec.	
Annealing	56	30 Sec.	30
Extensions	72	1 Sec.	
Final extensions	72	5 Min.	1

Temp: temperatures, Min: minutes, Sec: seconds.

All strains of bacteria were examined for virulence-associated genes (InlJ, InlA, *hlyA*, and *actA).* The primers and it is sequences were listed in Table 3.

Table 3: Target genes and primer sequences used.

Target genes	Sequences (5'-3')	Product size (bp)	Reference
InlJ-R	TGTAACCCCCGCTTACACAGTT	238	Liu, et al. 13
InlJ-F	AGCGGCTTGGCAGTCTAATA		
InlA-R	ACGAGTAACGGGACAAATGC	800	Liu, et al. 13
InlA-F	CCCGACAGTGGTGCTAGATT		
hly -R	GCCTGCAAGTCCTAAGACGCCAATC	707	Hudson ,et al. 14
hly-F	CTTGCAACTGCTCTTTAGTAACAGC		
actA-F	CGCCGCGGAAATTAAAAAAAG	890	Suárez ,et al. 15
actA-R	ACGAAGGAACCGGGCTGCTAG		

The PCR technique program for the *InlJ, InlA, and* hlyA and actA genes were studied as follows: initial denaturation at 94°C for five minutes, followed by thirty cycles of denaturation at 94°C for 30 seconds, annealing at 50 ,52 and 58°C for thirty seconds for *InlJ, InlA, and* hlyA and actA genes respectively, and extension at 72°C for 2.5 minutes for *InlJ, InlA* and for 1 minute for other genes, then extension at 72°C for seven minutes for all genes. Final extension at 10°C for ten for four genes minutes.

PCR technique amplification products were analyzed electrophoretically on a 1% horizontal agarose gel.[16]

Statistical Analysis

The results were analyzed using Chi-square tests (STATA v.14.0) at confidence level 95%.

RESULTS

L monocytogenes was molecular detected in a total of 61 (20.3%) aborted fetal organs, distributed as: 35 (57.4%), 15 (24.6%) and 11 (18.0%) strains from fetal brain, abomasal content and blood respectively (Table 4), (Figure 1).

Table 4: Percentages of aborted fetal specimens positive for *L. monocytogenes* in Nineveh governorate, Iraq

Type of samples	Number of examined	Number of positive	%
Brain	100	35	35*
Abomasal content	100	15	15
Blood	100	11	13
Total	300	61	20.3
Type of samples	100	35	35*

* Significantly high in comparison to other specimens at P<0.05

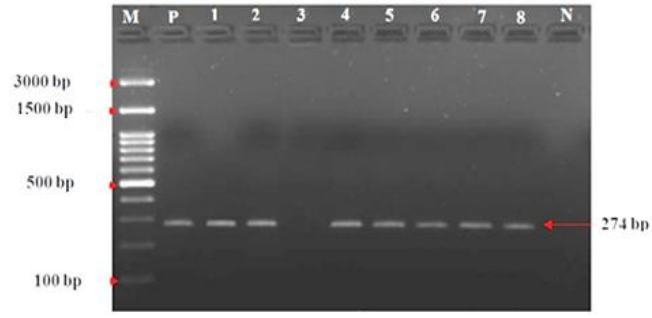

Figure 1: Electrophoresis and ethidium staining showing as a result of PCR procedures for *Listeria monocytogenes*, M: Represent marker. (1-8): Form positive result for *L.* monocytogenes with band size 274 bp, P: Positive control (L. monocytogenes ATCC: 7644), N: Negative control.

All *L. monocytogenes* strains were positive for three genes (*InlJ, InlA*, and hlyA) except for actA gene, detected only in 46 (75.4%) strains (Figs 2-4). Two abortigenic *L. monocytogenes* strains (HMB1 listeriolysin, HMB 2 listeriolysin) deposited in GenBank under accession numbers LC769365.1, and LC769366.1, and accession numbers of abortigenic two *L. monocytogenes* strains were positive for virulence-associated gene were deposited in the GenBank online database (Table 5).

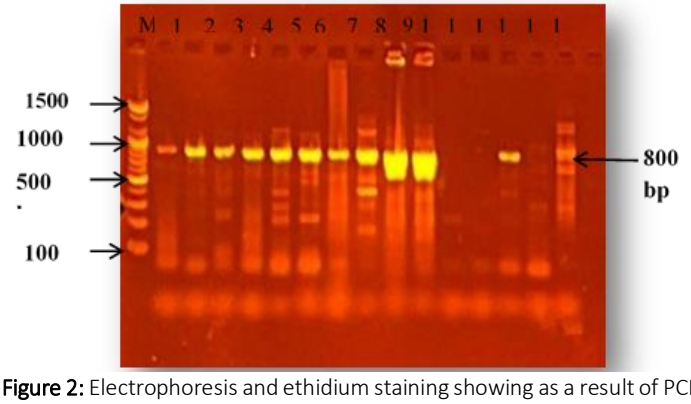

Figure 2: Electrophoresis and ethidium staining showing as a result of PCR procedures for Listeria monocytogenes Inter A., M: Represent marker (1-15) form positive result for Int A. with band size 800 bp.

Table 5: Strains and accession numbers of the L. monocytogenes isolated from aborted sheep fetuses in the Nineveh governorate, Iraq.

Strains	Accession numbers	Sources
HMBJ1 internalin J gene	LC769367.1	Aborted fetus blood-sheep
HMBJ2 internalin J gene	LC769368.1	Aborted fetus blood-sheep
HMBinlA1 internalin A gene	LC769373.1	Aborted fetus blood-sheep
HMBinlA2 internalin A gene	LC769374.1	Aborted fetus blood-sheep
HMBLLO1 listeriolysin O gene	LC769369.1	Aborted fetus blood-sheep
HMBLLO 2 listeriolysin O gene	LC769370.1	Aborted fetus blood-sheep
HMBactA1 actin-assembly inducing protein precursor gene,	LC769371.1	Aborted fetus blood-sheep
HMBactA2 actin-assembly inducing protein precursor gene,	LC769372.1	Aborted fetus blood-sheep

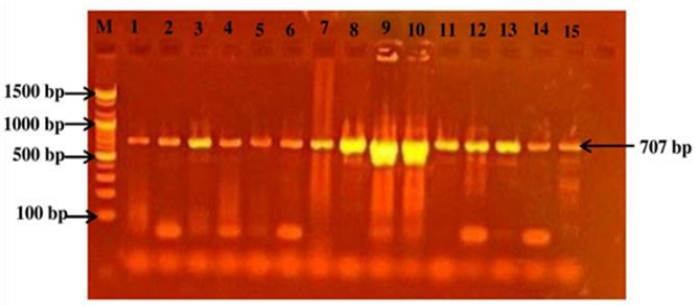

Figure 3: Electrophoresis and ethidium staining showing as a result of PCR procedures for Listeria monocytogenes Listerio Lysin O., M: Represent marker (1-15) form positive result for Listerio Lysin O. with band size 707 bp.

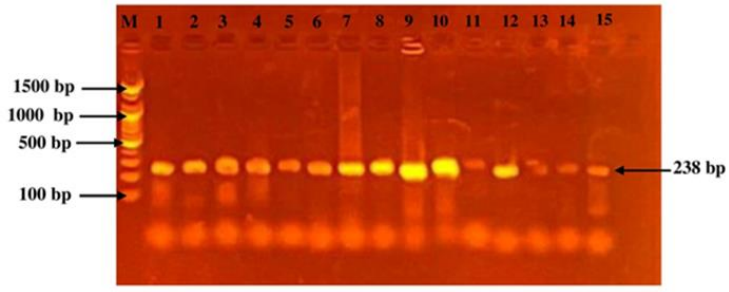

Figure 4: Electrophoresis and ethidium staining showing as a result of PCR procedures for Listeria monocytogenes Int J, M: Represent marker (1-15) form positive result for Int J. with band size 238 bp

DISCUSSION

Abortion in ruminants remains a difficult issue worldwide. It can result in unprofitable loss for farmers and an problematic of public health.[1] Abortion can be multicausal agents, and an extensive diagnostic research is essential to reach the accurate diagnosis[2].

In this study, we utilized molecular tool, for detection of abortigenic *L. monocytogenes*. Different procedures have been used to identify listeriosis, involving bacteriological, molecular and serology analyses.[17,18] Molecular approaches for identifying *L. monocytogenes* are becoming more frequently utilized because they are exceedingly reliable, and have high differentiation power within and between organisms that exhibit similar characteristics compared to cultural methods,[19] while the ordinary known microbiological methods routinely used for isolating *L. monocytogenes* in different samples usually need binary enrichment steps (enriched with Listeria selective supplements) which are later inoculated on the surface of the selective Listeria agar.[7] Additionally, *L. monocytogenes* are auxotrophic for seven amino acids including leucine, isoleucine, valine, methionine, arginine, cysteine, and glutamine and the bacteria also require four additional vitamins including riboflavin, thiamine, biotin, and thioctic acid[6]. After examination of 300 specimens were collected from ovine aborted fetuses, we identified *L. monocytogenes* DNA in 61 (20.3%), distributed as: 35 (57.4%), 15 (24.6%) and 11 (18.0%) strains from fetal brain, abomasal content and blood specimens respectively. The identification of clinical and ecological isolates of *L. monocytogenes* is significant since the same type has been shown to circulate within farms or geographical zones. *L. monocytogenes* genotypes related with human outbreaks were identified in dairy cows; thus, characterization of listerial isolates has implications for public health.[20]

Abortigenic *L. monocytogenes* strains in Iraq have not been studied with molecular characterization. The presence of *L. monocytogenes* has been identified serologically in 19.7% of camels in Kirkuk city, Iraq.[21] and in 11.5% in sheep flocks in Nineveh governorate, Iraq.[22]

In general, Listeria findings are many times smaller than our results. Researchers in Brazil, testing using molecular methods, found that 4 or 6.25% of the 64 materials analyzed were positive for *L. monocytogenes*.[23] Similarly Shoukat et al.[24] was detected of *L. monocytogenes* in 2.83% of aborted ewes in Kashmir Region, India, and 8.3% in Denmark.[25] Likewise, *L. monocytogenes* was isolated from aborted ewes in in Sharkia Governorate, Egypt[26], and thirty-one *Listeria* isolates out of 240 samples were recovered from diseased sheep with a prevalence rate of 12.9% in Egypt.[27]

These present findings of 20.3% as *L. monocytogenes* is slightly lower than what was reported by Wagner et al.[28],

who reported listerosis in 25% of aborted ewes in Austria. There may be many reasons for this variation in the results, including differences in diet type, specimen counts, and geographic location[1,3].

All *L. monocytogenes* strains were positive for three genes (*InlJ, InlA, and* hlyA) except actA gene was detected in 46 (75.4%) strains. The presence of two virulence genes in most *L. monocytogenes* isolates indicates that these isolates are virulent and can cause disease.[5]

A previous study also reported that the prevalence of hlyA gene among isolates was as high as 98.4%, while the prevalence of other virulence genes iapA, plcA, and plcB were 85.7%, 73%, and 68.2%, respectively.[29] In contrast to this study, Laximan et al.,[30] targeted virulence cluster genes (hlyA, iap, plcA, actA, and prfA) to identify *L.monocytogenes* in milk samples.

In conculsion, this study indicated that *L. monocytogenes* could be a noteworthy pathogen associated with ovine abortion cases in Nineveh governarate, and most of the brain specimens were positive for listeric infection compared to the other specimens.

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