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Role of *Listeria monocytogenes* in abortion of ewes

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Abstract

Abortion in ewes is a major problem threat their production caused by several pathogens, one of which in *L.Monocyotgenes*. A total of 150 samples of Placenta, Vaginal swabs and Foetal organs (50 each) were collected from aborted ewes in some sheep farms as many cases of abortion occurred to examine the presence of Listeria species. Listeria isolates were further identified by VITEK-2 system and confirmed by Polymerase Chain Reaction (PCR). Listeria spp. was isolated from 15 (10%) of the 150 samples, distributed as: 4 (8%), 1 (2%), and 10 (20%) isolates from Placenta , Vaginal swabs and Foetal organs, respectively and all isolates were identified as *L. monocytogenes*. Moreover, some other bacterial species were also isolated from few cases including *Staph. aureus* and *E.coli*. Among the 15*L. monocytogenes* isolates, 14 showed the presence of *hlyA*, and all isolates showed the presence of *inlA* virulence-associated genes.

L. monocytogenes isolates showed high resistance againstoxacillin, gentamicin, vancomycin, daptomycinand doxycylin (66.7%) followed by rifampicin and ciprofloxacin (53.3%), and, to a lesser extent, clindamycin (47%) and tetracycline (40%).whereas high susceptibility was shown toward ampicillin, levofloxacin, moxifloxacin, linezolid, and tigecycline (100%, each). Furthermore, 70% of *L. monocytogenes* isolates showed multi-resistance to at least four or more of the antibiotics tested. The findings of this study show that the contamination of examined sheep farms with *L. monocytogenes* is relatively high, and highlight the emergence of antimicrobial multi-drug resistant *L. monocytogenes* strains in diseased cases.

Keywords: *Abortion, Listeriamonocytogenes, ewes.*

Introduction

Listeric infections, caused by micro-organisms of the genus Listeria, occur worldwide and in a variety of animals, including humans. Listeria monocytogenes and L. ivanovii are the most important animal pathogens within the genus Listeria. Manifestations of listeriosis in animals include meningitis, encephalitis, meningoencephalitis, abortion and neonatal sepsis **(Arumugaswamy and Gibsone 1999)**. Abortion in sheep due to listeriosis is known to be an important problem in many sheep-raising areas of the world **(Chand *et al.*,1999**). Apart from L. monocytogenes, L. ivanovii, formerly known as L. monocytogenes serotype 5, is the only other pathogenic Listeria species **(Sergeant *et al.,* 1991).** Infection with L. ivanovii is recognised as a cause of abortions and stillbirths in sheep, as well as of the birth of live lambs that are weak and often fail to survive **(Sergeant *et al.,* 1991).**

Listeria monocytogenes is a facultative intracellular Gram-positive pathogen that causes serious disease in animals and human **(Baer, Miller & Dilger, 2013).** It is widely distributed in the environment and has been isolated from animal feces, food, and feed processing plants **(Gandhi & Chikindas, 2007).** *L. monocytogenes* can cause severe listeriosis infections, resulting in encephalitis, meningitis, septicaemia, abortion, premature birth, and stillbirth

(Selby *et al.,* 2006 and Silk *et al.,* 2012).

Mortality due to listeriosis ranges from 20 – 30% (**Lukinmaa *et al.,* 2003),** while in young animals with weakened immune systems, such as lambs and calves the mortality rate is up to 75% **(Amagliani *et al.,* 2004 and Jalali & Abedi, 2008).**

Virulence genes, which play a significant role in *L. monocytogenes* pathogenicity, have been described, including internalins encoded by *inlA*, listeriolysin O encoded by *hlyA* **(Liu *et al.,* 2007; Vazquez-Boland *et al.,* 2001).** Characterization of more than one virulence gene in *L. monocytogenes* isolates could be a rapid method to differentiate virulent from avirulent strains of the pathogen **(Liu *et al.,* 2007; Rawool *et al.,* 2007).**

L. monocytogenes is usually susceptible to antibiotics that are active against Gram-positive bacteria, except for cephalosporins, fluoroquinolones, and fosfomycin (**Charpentier & Courvalin, 1999).** However, the excessive use of antimicrobials has led to the emergence of antimicrobial-resistant bacteria **(Marian *et al.,* 2012; Safdar & Armstrong, 2003).** The levels of resistance are varied and influenced by antimicrobial use in animals, as well as geographical differences. Therefore, it is necessary to monitor the antibiotic susceptibility andresistance patterns of *L. monocytogenes* in diseased animals to establish an efficient control system for this field problem.

So the present study was conducted to:

Determine the occurrence of *L. monocytogenes* in some sheep farms and its role in abortion of ewes.

Identify the presence of virulence-associated genes in *L. monocytogenes* isolates.

Evaluate the antimicrobial resistance of *L. monocytogenes* isolates in order to recommend successful therapy.

2. Materials and Methods

2.1. Sampling

A total of 150 samples including Placenta (mostly cotyledons), Vaginal swabs and Foetal internal organs (50 each) were collected from three Brucella free sheep farms suffering from abortion of ewes at third trimester of gestation period in Sharkia Governorate, Egypt. All samples were transported in ice boxes to the laboratory within two hours of collection.

2.2. Isolation and identification of *Listeria spp.*:

Isolation and identification of *Listeria spp.* were carried out according to the US Food and Drug Administration (FDA) protocol **(Hitchins, 1992).** Briefly, samples were pre-enriched by inoculation in buffered peptone water (Himedia Lab, Mumbai, India) and incubated at 37 °C for 48 h aerobically; then 5 mL from each incubated pre-enriched sample was added to 10mL of *Listeria* enrichment broth (Himedia Lab, Mumbai, India) and incubated at 30°C for 48 h. A loopful of the incubated enriched broth was streaked directly onto Oxford agar (Himedia Lab, Mumbai, India), and incubated at 37°C for 24-48 h. Three to four presumptive *Listeria* colonies were grown overnight at 35 °C for 24–48 h on tryptic soy agar yeast extract (TSAye) ager. Morphologically typical colonies were verified by Gram staining and identified by VITEK-2 compact system (biomerieux, Marcy l'E toile, France). *Listeria* spp. isolates were adjusted to a McFarland standard of 0.50 in 0.45% saline solution then inoculated into the appropriate VITEK identification strip. The time between preparation of the solution and filling of the card was always less than 1 h. The analysis was done using the identification cards for-Gram-positive bacteria and Gram-negative card for Gram-negative staining bacteria, which are automatically read every 15 min. Data were analyzed using the VITEK-2 software version VT2-R03.1according to the manufacturer's instructions (bioMe´rieux, 2015). Samples were also cultured onto blood agar to investigate other possible causes of abortion.

2.3. Molecular characterization of *L. monocytogenes:*

The genomic DNA was extracted from biochemically confirmed suspected *L. monocytogenes* isolates using the QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany) and were subjected to PCR using primers specific for 16S rRNA for the amplification of 553 bp amplicons (**Abu Al-Soud & Radstrom, 1998).** Isolates identified as *L. monocytogenes* were

further subjected to molecular identification of *inlA* **(Liu *et al.,* 2007)** and *hlyA* **(Deneer and Boychuk, 1991)** virulence-associated genes. A positive control of L. monocytogenes was run alongside the tested isolates; this was kindly donated by the Biotechnology Unit, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt.

Table (1). Oligonucleotide primers sequences Source: Metabion (Germany):

Target gene	Sequence	Amplified product bp	Reference
16S rRNA	GGACCGGGG CTA ATA CCG AAT GAT AA F	1200 bp	**Kumar *et al.,* (2015)**
	TTC ATGTAGGCGAGTTGCAGC CTA R		
inlA	F ACG AGT AAC GGG ACA AAT GC	800 bp	**Liu *et al.*, (2007)**
	R CCC GAC AGT GGT GCT AGA TT		
hlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA F	174 bp	**Deneer and Boychuk, (1991)**
	R TGT-CAC-TGC-ATC-TCC-GTG-GT		

Table (2). Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No.RR310Akit:

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5µl
PCR grade water	5.5 µl
Forward primer(20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	5µl
Total	25 µl

Cycling conditions of the primers during cPCR Temperature and time conditions of the three primers during PCR are shown in Table (3) according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit.

Table (3). Cycling conditions of the different primers during cPCR:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16S rRNA	94˚C 5 min.	94˚C 30 sec.	60˚C 1 min.	72˚C 1 min.	35	72˚C 12 min.
inlA	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
hlyA	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

Antimicrobial susceptibility test:
Antibiotic susceptibility of molecularly confirmed *L. monocytogenes* isolates was performed with the VITEK-2 compact system (bioM´erieux, Marcy-l´´Etoile, France), using the Gram-positive AST-P592 bacteria card. Briefly, bacterial isolates obtained from TSAys agar were suspendedin 3 ml of 0.45% saline solution, mixed by vortex, and adjusted to the turbidity of 0.5 McFarland standard and then inoculated into the AST card according to the manufacturer's instructions. The 17 tested antimicrobial agents (and minimum inhibitory concertation (MIC) ranges) were ampicillin (2 to 32 µg/ml), oxacillin (0.25 to 4 µg/ml), gentamicin (0.5 to 16 µg/ml), vancomycin (1 to 32 µg/ml), ciprofloxacin (0.5 to 8 µg/ml), levofloxacin (4 µg/ml), moxifloxacin (0.25 to 1 lg/ml), erythromycin (0.25 to 8 µg/ml), clindamycin (0.25 to 8 µg/ml), linezolid (0.5 to 8 µg/ml), daptommycin (8 µg/ml), doxycycline (16 µg/ml), tetracycline (1 to 16 µg/ml), tigecycline (1 µg/ml), nitrofurantoin (0.5 to 32 µg/ml), rifampicin (0.5 to 32 µg/ml), and trimethoprim-sulfamethoxazole (10 to 320 µg/ml). The *L. monocytogenes* isolates were categorized as sensitive, intermediate, or resistant to the tested antimicrobial agents.
The sensitivity of *L. monocytogenes* isolates to 15 of the 17 tested antimicrobial agents was established according to the *Staphylococci* criteria of the Clinical and Laboratory Standards Institute (CLSI, 2006), based on the Conter, Paludi, Zanardi, Ghidini, Vergara &Ianieri (2009) protocol. The breakpoints for sensitivity to ampicillin and trimethoprim-sulfamethoxazole were defined according to the specific CLSI criteria **(CLSI, 2006).**

3-Results
Results are illustrated in tables (4-6) and photos (1-3).

Table (4). Distribution of Listeria spp. and other bacterial isolates isolated from Placenta , Vaginal swabs and Foetal organs examined samples

Sample source	Number examined	*Listeria spp.* positive	VITEK-2	System	
Placenta	50	4	4 (8%)	1 (2%)	2 (4%)
Vaginal swabs	50	1	1 (2%)	0 (0%)	3 (6%)
Foetal organs	50	10	10 (20%)	2 (4%)	4 (8%)
Total	150	15	15 (10%)	3 (2%)	9 (6%)

% calculated according to the number of examined samples in each item

Table (5) Results of cPCR Confirmation of *L. monocytogenes* isolates and detection of their virulence genes:

Strain no	Tested organs		
	16S Rrna	*inlA*	*hlyA*
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	+	+	+
14	+	+	-
15	+	+	+

Photo (1) illustrated results of cPCR Confirmation of *L. monocytogenes* isolates (16s RNA):

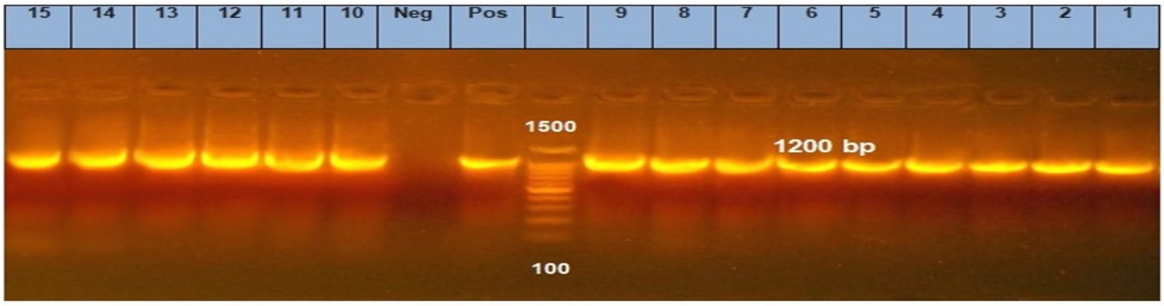

Photo (1): agarose gel elecotrophoresis showing : L:100 bp ladder
POS: +ve control
Neg: -ve control
Lanes : 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: (+ve) showing amplification of 685 bp fragments.

Photo (2) illustrated results of virulence gene (*hlyA)* detection in L. monocytogenes isolates:

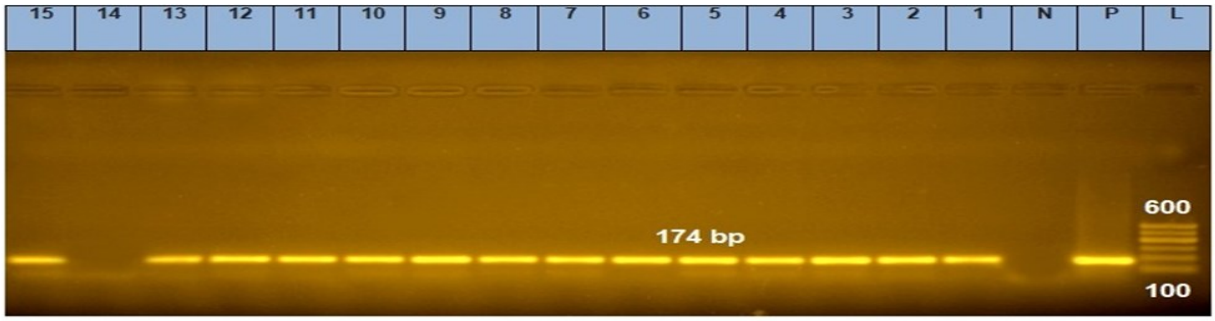

Photo (1): agarose gel elecotrophoresis showing : L:100 bp ladder
POS: +ve control
Neg: -ve control
Lanes: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15: (+ve) showing amplification of 685 bp fragments.
Lane 14: showing no amplification: negative.

Photo(2) illustrated results of virulence gene (*inlA*) detection in L. monocytogenes isolates**:**

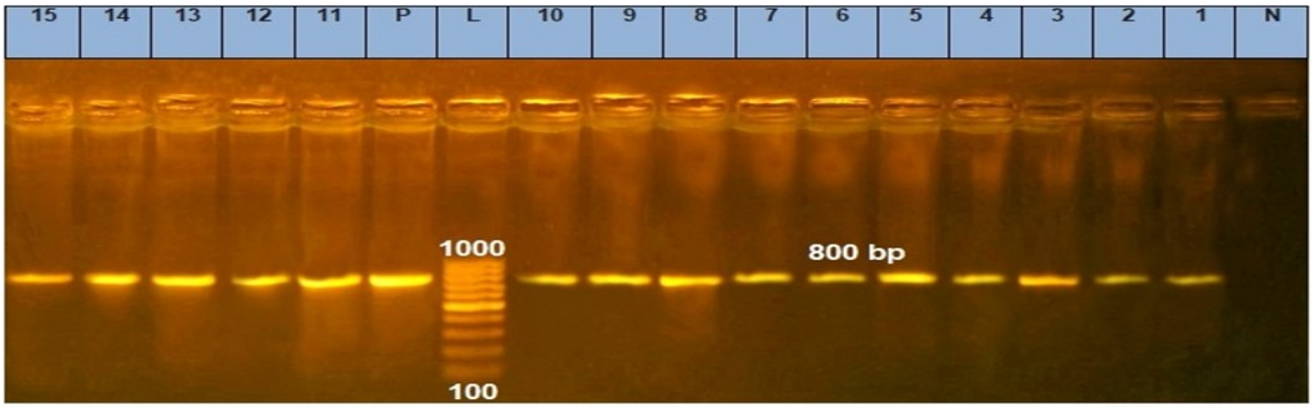

Photo (1): agarose gel elecotrophoresis showing : L:100 bp ladder
POS: +ve control
Neg: -ve control
Lanes: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: (+ve) showing amplification of 685 bp fragments.

Table (6). The antibiotic susceptibility profile of L.monocytogenes isolates (n = 15) recovered from placenta, vaginal swabs and aborted foeti internal organs**:**

Antibiotic	MIC range (µg/ml)[a]	Number (%) of isolates		
		Resistant	Intermediate	Susceptible
Ampicillin	2-32	0 (0)	0 (0)	15 (100)
Oxacillin	0.25-4	10 (66.7)	0 (0)	5 (33.3)
Gentamicin	0.5-16	10 (66.7)	2 (14)	3 (20)
Vancomycin	1-32	10 (66.7)	0 (0)	5 (33.3)
Ciprofloxacin	0.5-8	8 (53.3)	2 (14)	5 (33.3)
Levofloxacin	4	0 (0)	0 (0)	15 (100)
Moxifloxacin	0.25-8	0 (0)	0 (0)	15 (100)
Erythromycin	0.25-8	0 (0)	2 (14)	13 (86)
Clindamycin	0.25-8	7 (47)	0 (0)	8 (53.3)
Linezolid	0.25-8	0 (0)	0 (0)	15 (100)
Daptomycin	8	10 (66.7)	5 (33.3)	0 (0)
Doxycycline	16	10 (66.7)	5 (33.3)	0 (0)
Tetracycline	1-16	6 (40)	3 (20)	6 (40)
Tigecycline	1	0 (0)	0 (0)	15 (100)
Nitrofurantoin	0.5-32	0 (0)	2 (14)	13 (86)
Rifampicin	0.5-32	8 (53.3)	7 (47)	0 (0)
Trimethoprim-Sulfamethoxazole	10-320	0 (0)	2 (14)	13 (86)

[a] MIC: minimum inhibitory concentration
% calculated according to the number of listed L.momocytogenes isolates (15)

Discussion

Listeriosis is a sporadic disease of several animal species and humans, but it is most important economically in animals. Moreover, listeriosis is one of the most common causes of meningitis in humans and nonhuman primates . Listeric abortions are commonly caused by L. monocytogenes and occur in ruminants and many other species of domesticated animals. L. ivanovii is also recorded as a cause of abortion in sheep **(Arumugaswamy *et al.,* 1999)**

In the present study, *L. monocytogenes* was the main cause of abortion. Listeria spp. was isolated from 15 (10%) of the 150 samples, including 4 (8%), 1 (2%), and 10 (20%) isolates from Placenta , Vaginal swabs and Foetal organs , respectively all list spp. isolates were identified as L. monocytogenes also some other bacteria) E.coli -Staph aureus were isolated in few case 3 (2%)and 9(6%) respectively). Nearly similar results were recorded by (**Arumugaswamy *et al.,* 1999**) who recorded that.

Listeria isolates identified as *L. monocytogenes* by VITEK-2 system were also positive, using the PCR assay.
The presence of two virulence genes in almost of the *L. monocytogenes* isolates suggests that these isolates are virulent and can cause disease **(Jiang *et al.,* 2006; Van Stelten *et al.,* 2010)**

The antibiotic susceptibility test of virulent *L. monocytogenes* isolates (Table 6) revealed somewhat high resistance against oxacillin, gentamicin, vancomycin, daptomycinand, doxycylin (66.7%) followed by rifampicin and ciprofloxacin (53.3%), and, to a lesser extent, clindamycin (47%) and tetracycline (40%). Furthermore, 70% of *L. monocytogenes* isolates showed multi-resistance to at least four or more of the antibiotics tested. The high resistance of *L. monocytogenes* to tetracycline was previously reported in Egypt **(Khedr *et al.,***

2016) and could be attributed to the widespread use of tetracycline for treatment of infectious diseases in sheep farms in Egypt. Further, several studies have reported high prevalence of resistance to tetracycline in developed countries **(Jamali *et al.,* 2013; Rahimi *et al.,* 2010; Ayaz & Erol, 2010; Marian *et al.,* 2012).** While, a low prevalence of resistance to tetracycline was reported by Arslan and Özdemir, (2008). On the other hand, high susceptibility to ampicillin, levofloxacin, moxifloxacin, linezolid, and tigecycline (100%, each) was detected in the present study, similar to results in other reports (**Rodas-Suarez *et al.,* 2006; Wang *et al.,* 2013; Su *et al.,* 2016).**

Since the first report of antibiotic-resistant strains of *L. monocytogenes* **(Poyart-Salmeron *et al.,* 1990),** strains resistant to one or more agents have been found **(Conter *et al.,* 2009; Yan *et al.,* 2010; Zhang *et al.,* 2007).**

Likewise, in the present study, 70% of *L. monocytogenes* isolates showed multi-resistance to at least four of the antibiotics tested. Similar findings were observed by **Wang *et al.,* (2013),** who reported that 72.3% of *L. monocytogenes* isolated were resistant to at least three of the antibiotics tested. However, a low prevalence (<20%) of multi-resistant *L. monocytogenes* hasbeen observed in other studies **(Jamali *et al.,* 2013; Rahimi *et al.,* 2010).**

5. Conclusions and recommendations

-*Listeria* ***monocytogenes*** causes many disease problems in sheep herds mainly abortion in the third trimester of gestation period most of isolates harbourd virulence genes as *hlyA*, and *inlA* which are responsible for the pathogenicity of the organism, moreover some isolates showed multidrug resistence to the commonly used antibiotics.

-Strict hygienic, biosafety, biosecurity measure should be adopted in farms in order to facilitate disease control.

-Massive antibiotic (choosen after antibiograme) therapy application in infected herds in order to control infection and avoid miss use of antibiotics.

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