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Listeria monocytogenes virulance genes associated with keratoconjunctivitis in buffaloes

Ahlam, K.A. Wahba; Aliaa, A.E. Mohamed and Nahed, M.A. Shawky

Buffalo Diseases Research Department, Animal Health Research Institute, Dokki, Giza. Agriculture Research Center (ARC)

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Abstract

Listerial keratoconjunctivitis ('silage eye') is a wide spread problem in ruminants causing economic losses to farmers and impacts negatively on animal welfare. It results from direct entry of *Listeria monocytogenes* into the eye, often following consumption of contaminated silage. The bacteriological analysis of 75 conjunctival swabs from diseased buffaloes in a farm in El-Fayoum governorate reared indoor and feed on silage revealed 11 isolates resembling *Listeria* species (14.7%). Out of the 11 *Listeria* isolates, 5 isolates of *Listeria monocytogenes* (*L. monocytogenes*) (45.4%), 2 isolates of each *L. welshimeri* and *L. seeligeri* (18.2% each) and one isolate of *L. innocua* and *L. grayi* (9.1% each). The 5 *L. monocytogenes* isolates exhibited serological identification by agglutination test, synergistic hemolytic activity with beta toxin of *S. aureus* using CAMP test. In addition, the disease induced experimentaly by intraperitonial inoculation of mice and instillation in rabbit's eye with the isolated strains. All isolates were tested for antimicrobial susceptibility by disc diffusion method against group of antimicrobial agents of the most common used in the veterinary field. All tested isolates highly sensitive to ampicillin, erythromycin and amoxicillin; moderate sensitive to tetracycline and ofloxacin and resist against lincomycin. Polymerase chain reaction (PCR) was applied for detection of some virulence genes associated *L. monocytogenes* isolates (*inlA, inlB, prfA* and *hlyA* genes). PCR results revealed that, all isolates of *L. monocytogenes* were positive for the presence these genes that play the main role of pathogenesis of listeriosis leading to variety in clinical picture of the disease.

Keyword: *Buffaloes, conjunctivitis, Listeria monocytogenes and virulence genes.*

Introduction

Infectious keratoconjunctivitis is a highly contagious ocular infection affecting domestic and wild ruminants, and have major economic importance **(Brown *et al.,* 1998** and **Rajesh *et al.,* 2009)**. The condition is caused by a number of infectious agents in ruminants specially cattle, such as *Rhikettsiae*, *Chlamydia*, Viruses, *Mycoplasma* spp., *Neisseria catarrhalis, Moraxella bovis* and *L. monocytogenes* and characterized by epiphora, conjunctival inflammation, pigmental areas on the cornea, photophobia, blepherospasm, which might terminated by corneal ulceration and even panophthalmia **(Murat *et al.,* 2006)**. The disease is common in dairy herds, reducing milk production and body weight; however is rarely cause death **(Al-Gaabary *et al.,* 2008** and **Slatter *et al.,* 2008)**. It either transmitted by direct contact with infected materials from one animal to another or by indirect transmission of conjunctival exudates by flies **(John*et al.,* 2007)**, which identified as the main causative agents; however, other factors including ultra-violate light, concurrent diseases status, mechanical trauma and other ocular bacterial organisms may predispose to the infection **(Nightingale *et al.,* 2005**).

The genus *Listeria* includes 10 species out of these; *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and animals **(Guillet *et al.,* 2010)**. Listeria exists worldwide; furthermore, it has been isolated from soil, silage, faeces, sewage effluent, and stream water. It can survive for years in organic materials and is capable of proliferating at temperature from

3˚C to 45˚C **(Cocolin *et al.,* 2005; Ho *et al.,* 2007** and **Muchaamba *et al.,* 2019)**.
Listeriosis is an infectious disease caused by a Gram-positive asporogenic bacterium, *L. monocytogenes*, which is capable of intracellular replication **(Ray and Bhunia, 2013)**. It affects humans and over 60 species of domestic and wild animals including fish and birds **(Swetha *et al.,* 2013** and **Reda *et al.,* 2016)**. Septicemia, encephalitis or abortions are the most common clinical manifestations of infection **(Radostits *et al.*, 1994)**, but ocular symptoms may also be seen. The term "silage eye" refers to listerial kerato-conjunctivitis, and may involve uveitis with hypopyon and miosis **(Erdogan *et al.,* 2001** and **Pearce and Moore, 2013)**.

Ruminants are the most frequently affected domestic animals **(Cooper and Walker, 1998** and **Kotzamanidis *et al.,* 2019)**. Contaminated silage is classic source of infection; other sources include particularly organic refuse as poultry litter. Growth and multiplication of *L. monocytogenes* naturally present on ensiled plant material are enhanced in poorly fermented silage with high pH. Some poorly fermented silage linked to listeriosis cases in ruminants have shown Listeria counts as high as 10^8 colony-forming units (CFU)/g wet weight of silage **(Laven and Lawrence, 2006)**.

Although silage has been well established as a common source of systemic listeriosis infections in farm ruminants, it has also been hypothesized that Listeria-contaminated silage may be a source of listerial eye infections. For example, some reports noted that silage on farms with cases of listerial ocular infections was fed at or above the height of the animal's head, possibly leading to direct exposure of eyes to contaminated feed. In addition, it has been noted that most ruminant cases of listerial ocular infections appear to occur in the winter and early-spring months, when the animals are housed inside and fed silage **(Pauly and Tham, 2003** and **Nadeau, 2007)**.

*L. monocytogenes*is a bacterium that invades replicates and multiplies in a variety of mammalian cells, including macrophages, epithelial cells and fibroblast cells **(Hellenic Center for Disease Control and Prevention [HCDCP], 2018)**. A number of genes and gene products necessary for the intracellular survival of this pathogen have been previously reviewed **(Osman *et al.,* 2016)**. Major virulence factors include listeriolycin O (LLO) a toxin having pore-forming activity; *Act*A a factor responsible for polymerization of host actin; a group of internalin family proteins which play a major role in entry, namely, *inl*A and *inl*B; two phospholiphases, *plc*A and *plc*B; a metalloprotease, mpl and uhpT, a system for uptake of sugars **(Dussurget *et al.* 2004** and **Schnupf and Portnoy, 2007)**. UhpT helps bacteria to take up glucose-6-phosphate inside the cell, thus enhancing growth and multiplication **(Camejo *et al.,* 2011)**.

These virulence factors are under direct control of a transcriptional factor, *Prf*A **(Lecuit, 2007)**. A thermosensor controls this *Prf*A and, based on environmental temperature, this sensor's 5' UTR can take up different secondary structures **(De las Heras *et al.,* 2011)**. Hence, there is an optimal expression of *Prf*A at higher temperatures than at lower temperature where there is a down regulation of *Prf*A. As a result, all virulence genes are expressed to maximum at 37 C compared to lower temperatures **(Johansson *et al.,* 2002** and **Muchaamba *et al.,* 2019)**.

The hemolysin gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria* spp. Subsequent characterization of the *hly* locus led to discovery of the chromosomal virulence gene cluster in which most of the genetic determinants required for the intracellular life cycle of pathogenic *Listeria* spp. reside. The *hly* product, *hly*, was also the first virulence factor for which a precise role in the pathogenesis of *Listeria* infection was demonstrated. *Hly* is a key virulence factor essential for pathogenicity, having a vital role not only in intracellular parasitism but also in several other functions in the interaction of listeriae with their vertebrate host. Cell culture studies of the effects of *hly* inactivation showed that hemolysin is required for the survival and proliferation of *L. monocytogenes* within macro-

phages and nonprofessional phagocytes **(Muchaamba *et al.*, 2019)**.

Internalins (*inl*) are the protein products of a family of virulence associated genes found in pathogenic *Listeria* spp. The first members of this family to be characterized, *inlA* and *inlB*, encoded by the *inlAB* operon, were identified in *L. monocytogenes* by screening a bank of transposon-induced mutants for impaired invasiveness in Caco-2 cell monolayers. *InlA* was shown to function as an invasin, mediating bacterial internalization by these normally nonphagocytic epithelial cells, and was therefore named internalin**,** a large number of internalin homologs have since been identified in both *L. monocytogenes* and *L. ivanovii* **(Liu *et al.*,2007** and **Radoshevich and Cossart, 2018)**.
The present study planned to throw the light on *L. monocytogenes* as one of the causative agent of keratoconjunctivitis in buffaloes based on selective plating media, biochemical characterization, serological identification, there in vivo virulence potential, antibiotic resistance (ABR) profile and some virulence associated genes (*hly*A, *Int*A, *Int*B, and *Prf*A).

Materials and Methods

Collection of samples: A total of 75 conjunctival swabs were collected in the winter and early-spring months from a private buffalo farm in El-Fayoum governorate using separate sterile swabs by inserting into the conjunctival sac of animals showed symptoms of keratoconjunctivitis and feed on silage, gently rolled and then inoculated into Trypticase soya broth supplemented with 0.6% of yeast extract (TSB-YE); and transferred rapidly in ice box to the laboratory for bacteriological examination to investigate the incidence of *L. monocytogenes* according to **Sneath *et al.* (1989)** and **Quinn *et al.* (2011)**.

Cultivation and identification of *Listeria* species: For direct plating, swabs were streaked onto Palcam agar plates (Oxoid; CM0877B and SR0150E) media and incubated at 35°C for 24-48 hs. To detect the *listeria* cells even in low number, the ISO 112090-1 food sampling protocol was also used. The same swabs used for direct plating were placed in 20 ml of Fraser broth (Oxoid; CM0895B and SR0156E)) and vortexed for 30 s then a loopful from each broth were streaked onto Palcam agar plates and incubated as previously. The plates were examined for the presence of characteristic colonies presumed to be Listeria (grey green in color with a black sunken center and a black halo).

Confirmation of the *Listeria* isolates: All strains were tested for purity, in addition to morphological and biochemical characteristics. Colonies suspected to be *Listeria* were transferred onto Trypticase soya broth supplemented with 0.6% of yeast extract (TSB-YE) (Difco, Bacton, USA) and incubated at 30 °C for 18 to 24 h. Those putative *Listeria* colonies were characterized using Gram's staining, tumbling motility at 20–25 °C, catalase test, Methyl Red–Voges Proskauer (MR–VP) reactions, characteristics of haemolysis on 5 % sheep blood agar, carbohydrate utilization and CAMP test. The CAMP test was undertaken using *S. aureus* (ATCC 7494) and *Rhodococcus equi* (ATCC 6939) and *E. coli* (ATCC 25922). They were streaked in single lines across a sheep blood agar plate so that the two cultures were parallel and diametrically opposite. Test strains were then streaked at right angles and 1 to 2 mm apart to *S. aureus* and *R. equi.* Simultaneously, standard strain of *L. monocytogenes* (ATCC 7494) was streaked onto blood agar plates. The plates were then incubated at 37 °C for 18 to 24 h. The test culture streaks were tested for enhanced β-hemolysis at both ends proximal to the reference cultures. The zone of enhanced β-hemolysis may resemble an arrowhead, circle or rectangle. The presence of this zone indicates a CAMP-positive reaction. Absence of enhanced β-hemolysis indicates a CAMP-negative reaction. *L. monocytogenes* and *L. seeligeri* are CAMP-positive to the Staphylococcus reference strain and CAMP-negative to *R. equi*. In contrast, *L. ivanovii* is CAMP-positive to the *R. equi* reference strain and CAMP-negative to the Staphylococcus reference strains.

For the carbohydrate utilization test, isolated colonies from TSA-YE were transferred into test tubes containing xylose, rhamnose and

mannitol and incubated at 37°C for up to 5 days. Positive reactions were indicated by yellow color (acid formation) and occurred mostly within 24 to 48 h. In parallel, strains were identified using the API® Listeria system (bioMe'rieux, Marcy l'Etoile, France).

Serotyping of *L. monocytogenes* according to Federal Register (1988): Serotyping was carried out on the 5 *L. monocytogenes* isolates using commercial specific antisera (Behringwerke AG) against the serovars 1 and 4, following the manufacturer's instruction.

Laboratory animal pathogenicity tests (Federal Register (1988): The classical tests for *Listeria* pathogenicity are the Anton conjunctivitis test using rabbits and intraperitonial inoculation using mice.

a- *Using rabbit*: An experimental keratoconjunctivitis test (Anton's eye test) was performed in rabbits by inoculating by 0.1 ml (a drop) of inoclum having approximately 10^9 CFU of each *Listeria* isolate/ml using Macferland's onto the eye. Only *L. monocytogenes* causes purulent keratoconjuctivitis within 24–36 h of inoculation **(Fentahun and Fresebehat, 2012)**.

b- *Using mice*: The pathogenicity testing of the *Listeria* isolates by mice inoculation test was performed according to the method described by **Menudier *et al*. (1991)**. Briefly, 36 mice weighing 18–20 g were housed 3 per cage and allowed to acclimatize for one week. They were inoculated intraperitoneally with 0.1 ml (10^9 CFU) of each *Listeria* isolate. Three mice were used for each isolates and three mice were kept as control. The death rate and post-mortem changes as well as the reisolation of the organism from the internal organs and heart blood were recorded.

Determination of the in-vitro antimicrobial suceptibility test among *Listeria* isolates: According to the Clinical Laboratory Standards Institute **(CLSI, 2012)**, all *Listeria* isolates concluded the 5 isolates of *L. monocytogenes* was tested for antimicrobial susceptibility on Mueller Hinton agar by disc diffusion method and using the following Oxiod antibiotic disks: ampicillin (AMP10), amoxicillin (AX25), erythromycin (E15), tetracycline (TE20), gentamicin (CN10), ofloxacin (OFX5), penicillin G (P10), cephalexin (CL30) and lincomycin (L2). The plates were incubated at 35°C for 24 hours. The zone of inhibition around each disc was measured and the interpretation was made as per the zone size interpretation chart provided by the disc manufacturer.

Detection of virulence genes of *L. monocytogenes* isolates by Polymerase chain reaction (PCR): According to **Sambrook and Russell (2001),** the 5 isolates of *L. monocytogenes* were screened for the presence of *hly*A, *int*A, *int*B, and *prf*A genes as following:

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56^OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from Metabion (Germany) are listed in Table (1).

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp and 100 bp plus Ladders (Qiagen, Germany, GmbH) and generuler 100 bp ladder (Fermentas, Thermoscientific) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
HlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA TGT-CAC-TGC-ATC-TCC-GTG-GT	174	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Deneer and Boychuk (1991)**
PrfA	TCT-CCG-AGC-AAC-CTC-GGA-ACC TGG-ATT-GAC-AAA-ATG-GAA-CA	1052	94˚C 5 min.	94˚C 30 sec.	50˚C 50 sec.	72˚C 1 min.	72˚C 10 min.	**Dickinson *et al.* (1995)**
InlA	ACG AGT AAC GGG ACA AAT GC CCC GAC AGT GGT GCT AGA TT	800	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Liu *et al.* (2007)**
InlB	CTGGAAAGTTT-GTATTTGGGAAA TTTCATAATCGCCATC ATCACT	343	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

Results and Discussion

Listeriosis, also termed as silage disease, circling disease and meningoencephalitis, is caused by *Listeria monocytogenes*. It is an infectious and fatal disease of animals, birds, fish, crustaceans and humans, where septicemia and encephalitis are predominantly observed **(Wesley, 2007** and **World Organisation** for **Animal Health [OIE], 2014)**. The organism has an intracellular life cycle that can pass from cell to cell without release from the cell. This ability presents its potential to cross placental, blood brain and blood eye barriers, explaining its pathogenesis and clinical signs **(Janakiraman, 2008** and **OIE, 2014)**.

Domesticated ruminants probably play a key role in the maintenance of *Listeria* spp. in the rural environment via a continuous fecaloral enrichment cycle. Diseases due tolisterial infection usually occur in animals in winter and spring **(OIE, 2014)**. Three distinct principal clinical forms of listeriosis exist, which include septicemia, encephalitis, and abortion **(Kamal *et al.,* 2012)**; in addition to these more common manifestations of listerial infection, mastitis, keratoconjunctivitis, and uveitis have also been associated with *Listeria* infection in ruminants. Ocular signs in the neurological form of listeriosis include facial paralysis with ptosis, medial strabismus, nystagmus, and amaurosis **(Adel *et al.,* 2018)**. Uveitis with hypopion has been described in chronic cases of the disease **(OIE, 2014)**.

The ocular form in ruminants, without clinical signs of other forms, has been related to feeding contaminated silage in elevated feed bunkers and self-feed baled grass silage in housed cattle and sheep during the winter **(Åkerstedt and Hofshagen, 2004)**. Eye infections have been documented in sheep, cattle, fallow deer and humans which manifested by swollen, hyperemic conjunctivae, epiphora, photophobia, clouding of the cornea, and scattered white corneal foci **(Cullen and Webb, 2013** and **OIE, 2014)**.

A total of 75 conjunctival swabs collected from buffaloes suffering keratoconjunctivitis were screened for the prevalence of *Listeria* species. Identification of *Listeria* species on Palcam agar plates was based on aesculin hydrolysis and mannitol fermentation. All *Listeria* species hydrolyse aesculin as evidenced by a blackening of the medium. Mannitol fermentation was demonstrated by a colour change in the colony and/ or surrounding medium from red or gray to yellow due to the production of acidic end products. The selectivity of the Palcam medi-

um is achieved through the presence of lithium chloride, polymixin B sulphate and acriflavine hydrochloride present in the medium base and ceftazidime provided by Palcam antimicrobial supplement. These agents effectively suppress growth of most commonly occurring non-*Listeria* species of bacteria present in samples.

Table (2). Prevalence of *Listeria* species recovered from infected eye swabs:

Source of isolates	Sample's number	Prevalence of *Listeria species*	
		Positive sample	%
eye swabs	75	11	14.7

%: was calculated according to the total number of examined samples (75).

As shown in Table 2, the bacteriological examination revealed 11 isolates resembling *Listeria* spp. (11/75; 14.7%). nearly same results were also indicated by **Kamal *et al.* (2012)** who examined 360 conjunctival swabs from keratoconjunctivitis buffaloes which yielded *Listeria* species in a percentage of 14.3, higher rate were recorded previously by **(Al-Gaabary *et al.*, 2008**, **Yeruham *et al.*, 2001)** who recorded prevalence rate (16.9%), and mention that the variation of these rates may be attributed to the animal susceptibility, in addition to probability of exposure which governed by animal housing and management system, season and localities, thereby susceptibility were increased through some factors such as high stocking density, close confinement of animals to barns, movement of animals through dusty yards, the presence of flies which might consider as the main transmitter, movement of stock through long grasses, ultraviolet solar radiation, eye lid pigmentation and the presence of other pathogens.

Table (3) showed that, five isolates were identified as *L. monocytogenes* in a percentage of 45.4 %, two isolates were identified as *L. seeligeri* (18.2%), two isolates were identified as *L. welshimeri* (18.2%), and one isolate identified as *L. grayi* (9.1%) and also one isolate identified as *L. innocua* (9.1%), from all tested samples. These results achieved in Table (3) found that, *L.monocytogenes* was more prevalent identified serotypes from total *Listeria* isolates recovered, this explain the importance of such species in the pathogenesis of listeriosis. This opinion was fully supported by speculation of others as **Gebretsadik *et al.* (2011); Hanaa (2013); Al-Ashmawy *et al.* (2014)** and **Adel *et al.* (2018)**.

Table (3). Incidences and characteristics of *Listeria* species in the positive listerial eye swabs **(Sneath *et al.*, 1989 and Quinn *et al.*, 2011)**.

Listeria species (11)	No.	%	Hemolysis on sheep blood aga	CAMP Test		Acid production from sugars		
				SA	RE	D-mannitol	L-rhamnose	D-xylose
L.monocytogenes	5	45.5	+	+	-	-	+	-
L. seeligeri	2	18.2	+	+	-	-	-	+
L. welshimeri	2	18.2	-	-	-	-	-	+
L. grayi	1	9.1	-	-	-	+	-	-
L. innocua	1	9.1	-	-	-	-	-	-
Total	11	100						

No.: number of Listerial isolates %: was calculated according to the total number of Listerial isolates (11).
SA=*Staphylococcus aureus* RE= *Rhodococcus equi* + = Positive reaction - = negative reaction

L. monocytogenes is often detected in low numbers by cultivation, sometimes together with other bacteria. This observation is important for the bacteriologist when cultivating samples from cases of keratitis, and may be related to the intracellular growth of *L. monocytogenes.* The low number of reported cases, and also, as noted by other authors there may be lacking awareness among clinicians as well as bacteriologists regarding the potential role of *L. monocytogenes* in ocular infections **(Evans *et al.*, 2004)**.

According to **Groves and Welshimer (1977),** the CAMP test can be used to differentiate between hemolytic *Listeria* species; *L. monocytogenes*, *L. ivanovii* and *L. seeligeri***.** As observed in Table (3), CAMP test was useful for detection of synergistic reactions of hemolysis of *L. monocytogenes* and *L. seeligeri* with Beta toxin of *S. aureus* only through enhances the lysis of sheep erythocytes on blood agar by a synergistic effect between a phospholipase and listeriolysin of each *L. monocytogenes* and *L. seeligeri* and a sphingomyelinase of *S. aureus* as observed by **Mckellar (1994)**. All virulent *Listeria* (mostly *L. monocytogenes*) are hemolytic **(Rocourt *et al.*, 1983)** whereas non-hemolytic *Listeria* is avirulent. This observation led rather early to the assumption that hemolysin production is an important virulence factor in *Listeria* infections. The organism show hemolytic activity due to a single polypeptide molecule, listeriolysin O (LLO). Listeriolysin O has been implicated as a main component of virulence in *L. monocytogenes* **(Isome *et al.*, 1995)**. *L. monocytogenes* colonies can be detected in a majority of other listeria on the basis of hemolysis **(Beumer *et al.*, 1997**

The recorded results in Table (4) showed that, out of the 5 *L. monocytogenes*, 2 isolates could be identified serologically as *L. monocytogenes* serotype 2/1a, 2 isolates could be identified serologically as *L. monocytogenes* serotype 2/1b and the remaining isolate were identified serologically as *L. monocytogenes* serotype 4b by using commercial specific antisera (Behringwerke AG) against the serovars 1 and 4 and following the manufacturer's instructions.

Table (4). Serotyping of *L. monocytogenes* strains.

Species	Total number of Isolates	Serotypes					
		Type 2/1A		Type 2/1B		Type 4B	
		No.	%	No.	%	No.	%
L. monocytogenes	**5**	2	40	2	40	1	20

No: Number of positive serotype %: Was calculated according to the total number of isolates (5).

It was noticed that, *L. monocytogenes* Type 2/1a and 2/1b was the most prevalent serogroups recovered from the samples with an incidence of 40% for each, meanwhile Type 4b was recovered with an incidence of 20 % by the available antisera, further evidence comes from the fact that only 3 of the 12 known serovars of *L. monocytogenes*, 1/2a, 1/2b, and 4b, account for more than 90% of human and animal cases of listeriosis **(Farber and Peterkin, 1991)**. Physical locations suggest that, the inflamation of eye in current study originated in the feed. Although *L. monocytogenes* was not isolated from the silage, the identified *L. monocytogenes* serotypes are commonly isolated from silage **(Jessica *et al.*, 2015)**. **Erdogan (2010)** observed that, there are a strong correlation has been established between silage eye and the use of big bale silage and silage feeding in ring feeders and these infections are believed to occur when the organism directly enters the eye, possibly facilitated by corneal abrasions.

Also **Nadeau (2007)** noticed that, the high pH of the barley silage makes this a likely source.

Barley silage can be difficult to ferment correctly, particularly if a maturecutting is used, as the water content is often too low for effective fermentation. It is tempting to speculate that drier silage may predispose to the development of oral lesions, thus, potentially providing entry points for *L. monocytogenes* in the feed. Also our results go ahead with **Pauly and Tham (2003)** who reported that**,** high ash content of silages indicates also the contamination with soil, which is the major route of the contamination of silages by *L. monocytogenes*. **Sarigson (1993)** noticed that, *L. monocytogenes* present in poorly prepared silage multiplies and is the most common source of infection for cattle.

The association between *Listeria* species and the virulence has been made by using animal model. Mice and rabbits are the animals most frequently used to study *Listeria* infection in the laboratory. **Quinn *et al.* (2011)** concluded that, most *L. monocytogenes* isolates of animal origin are virulent, a characteristic which can be confirmed by instillation of a drop of broth culture into the eye of the rabbit to induce keratoconjunctivitis (Anton's test). Also **Vanderzant and Splittstoesser (1992)** indecated that, experimentally strains of *L. monocytogenes* differ widely in virulence as do the strains of other *Listeria* species, and added that, the pathogenicity within genus *Listeria* is restricted to two species *L. monocytogenes* and *L. ivanovii.*

In current study the pathogenicity of *L. monocytogenes* isolates was investigated in rabbits and mice. Among rabbit's pathogenicity test, attempt were made to infect 11 rabbits by all *Listeria* species isolated by instilling a live bacterial suspension into the conjunctiva. A purulent conjunctivitis (and occasionally a keratitis) developed within 24-72 h as shown in Tables (5), all isolates of *L. monocytogenes* proved to be highly pathogenic to rabbits and showed different degree of conjunctivitis. No reaction could be observed in rabbits infected with *L. seeligeri, L. welshimeri, L. grayi* and *L. innocua*. These observations is in agreement with the observation made by **Osman *et al.* (2016)** who observed that, instillation of a pure culture of *L. monocytogenes* into the conjunctiva of rabbit gave rise to severe conjunctivitis within 24h followed by keratitis and the animal itself rarely dies.

Table (5). Experimental infection of rabbit with *Listeria* species strains by eye instillation (Anton's test).

Number of examined strains ($1x10^{9}$ bacterial cell)	**Anton's test**					
	+++		++		+	
	Positive number	**%**	**Positive number**	**%**	**Positive number**	**%**
***L. monocytgenes* (5)**	2	40	2	40	1	20
Other *listeria* species (6) *(L. seeligeri, L. welshimeri, L. grayi* and *L. innocua).*	-	0	-	0	-	0

%: was calculated according to the number of examined strains.
+++: Severe conjunctivitis appeared within18 hours
+: Weak conjunctivitis after 48 hours
++: Conjunctivitis after 24 hours

The production of listeriosis for mice was investigated experimintally as another classical method for identification and demonstrating the virulance in listeria by intraperitonial injection of mice with ($1x10^{9}$ bacteria) of *L. seeligeri, L. welshimeri, L. grayi* and *L. innocua* (one isolate each) resulted in no death, no clinical signs or post-mortem finding in all injected mice. On the other hand, the 5 *L. monocytogenes* isolates caused death rate ranges from 33.3 to 100% in mice within 1-6 days after injected of 10^{9} bacterial cells as shown in Table

(6). Nearly similar observations were reached by **Quinn *et al.* (2011)** who recorded that, *L. monocytogenes* killed the mice withen 2-4 days, and added that, the LD_{50} for mice ranged from $3x10^2$ to $1x10^9$ bacteria depending on mouse used, route of inoculation and the strain of listeria used. *L. monocytogenes* could be reisolated from heart blood, liver, spleen as well as brain of the infected mice. Severe conjunctivitis, subcutaneous heamorage septicaemic organs and tiny foci of necrosis appeared among spleen and liver of dead mice these strongly suggest that, *L. monocytogenes* isolates were highly virulent among other *Listeria* species which come in agreement with the post-mortem examination of **Osman *et al.* (2016)**.

Table (6). Experimental infection of mice with the 5 examined *L. monocytogenes* and other *Listeria* species.

Number of examined strains ($1x10^9$ bacterial cell)	**Number of inoculated mice**	**No. of dead mice/No of exposed**		**Death of mice within (days)**
		Number of dead mice	**%**	
***L. monocytogenes* strains(5)**	3	3	100	4^{th}, 5^{th}
1	3	3	100	4^{th}, 5^{th}
2	3	2	66.7	5^{th}, 6^{th}
3	3	2	66.7	5^{th}, 6^{th}
4	3	1	33.3	6^{th}
5				
Other *Listeria* species(6) (*L. seeligeri, L. welshimeri, L. grayi* and *L. innocua*)	18 (3x6)	-	0	-

%: was calculated according to the number of inoculated mice.

Although the incidence of Listeriosis in Egypt is low, it required early diagnosis and appropriate antimicrobial therapy. For many years, there was no evidence of major changes in the susceptibility to antibiotics of *L. monocytogenes* isolated in industerialized countries **(Heger *et al.*, 1997** and **Osman *et al.*, 2016)**. In the present investigation, the in vitro sensitivity pattern of 11 *Listeria* isolates recovered from the examined infected buffalo eye to identify those antimicrobial agents to which *Listeria* species are generally susceptible to be useful in the treatment of listeriosis and to identify those antimicrobial agent which they are also resistance. As shown in Table (7), antimicrobial susceptibility tests were carried out using the disk diffusion method with nine different antibacterial drugs including penicillin (P10), ampicillin (AMP10), cefalexin (CL30), erythromycin (E15), tetracycline (TE20), gentamicin (CN10), lincomycin (B10), amoxicillin (Ax25**)** and ofloxacin (OFX). All tested *L. monocytogenes* and other *Listeria* species were highly sensitive to ampicillin (11/11), erythromycin (10/11), Amoxicillin (9/11), moderate sensitive to tetracycline (8/11) and ofloxacin (7/11) as shown in Table (7). On the other hand the major resistance phenotypes were found to be against lincomycin (0/11).

These recorded results agree with **Tobias *et al.* (2015)** and **Su *et al.* (2016)** who concluded that, Listeria is usually susceptible to a wide range of antibiotic including ampicillin and erythromycin, and also go hand in hand with **Raorane *et al.* (2014)** who observed that, all *L. monocytogenes* isolates were sensitive towards, erythromycin, oxytetracycline, ampicillin, doxycycline and ciprofloxacin and showed intermediate resistances towards the penicillin, gentamicin and vancomycin. In addition this is in accordance with the well-known fact that *Listeria* species particularly *L. monocytogenes* are resistance to cephalosprins as recorded by

Cabeza *et al.* (1989). Moreover **Radostits *et al.* (1994)** recordrd that, *Listerial* infections are commonly treated with penicillins or tetracylines, which may be effective in early stages of systemic disease. However, if severe symptoms have developed prior to initiation of intensive antibiotic treatment, the outcome is commonly fatal.

Also **Weber *et al.* (1999)** reported that, if the disease is left untreated, it can run up to three weeks and with systemic and local antibiotic treatment up to 10 days. However, in some cases, signs disappeared within two weeks after the onset of the treatment in severely affected animals and in mildly affected in 4 days without therapy, just by discontinuing feeding from big bales, cattle with ocular listeriosis spontaneously healed within a week after they removed the possible source of infection.

Table (7). Determination of the in-vitro antimicrobial suceptibility test among *Listeria* isolates.

Chemotherapeutic Agent	Number of senstive Isolates	Number of resistant isolates
Ampicillin AMP (25 µg)	11/11	0
Amoxicillin Ax (25 µg)	9/11	2/11
Erythromycin E (15 µg)	10/11	1/11
Tetracycline TE (30 µg)	8/11	3/11
Ofloxacin OFX (10 µg)	7/11	4/11
PenicillinG P (10 IU)	5/11	6/11
Cephalexin CL (30 µg)	4/11	7/11
Gentamycin CN (10 µg)	5/11	6/11
Lincomycin L (2µg)	0	11/11

Ability of *L. monocytogenes* to cause disease depends upon the expression of virulence factors and immune status of individuals **(Kocaman and Sarımehmetoglu, 2016)**. *L. monocytogenes* is one of the most invasive bacteria known and is capable of crossing intestinal**,** transplacental and blood brain barriers of the host, but the normal route of infection is by crossing intestinal barriers particular through the M cell of Payer's patches **(Gray and Killinger, 1996** and **Lecuit *et al*., 2004)**.

L. monocytogenes virulence involves host cell adhesion and invasion, cytosolic replication and motility, and cell-to-cell spreading **(Camejo *et al.,* 2011)**. Each stage depends on the activity of well-characterized virulence factors, acting by subversion of host cell functions **(Chen *et al.,* 2017)**. Within the host, *L. monocytogenes* parasitizes macrophages and invades non-phagocytic cells, utilizing its virulence factors to mediate cell-to-cell spread **(De las Heras *et al.,* 2011)**. **Liu (2006)** and **Liu *et al.* (2007)** recorded that, under normal circumstrance *L. monocytogenes* enters the host via contaminated food. After surviving exposure to host proteolytic enzyme, the acidic stomach environment (pH 2.0), bile salts and non-specific inflammatory assaults; *L. monocytogenes* adheres to and is internalized by host cells with the assistance of a family of surface proteins called internalins (e.g, *inl*A and *inl*B). Whereas *inl*A interacts with E-cadherin to mediate *L. monocytogenes* entry into epithelial cells, *inl*B recognizes C1q-R (or Met) to facilitate *L. monocytogenes* entry into a much broader range of host-cell types, including hepatocytes, fibroblasts and epithelioid cells **(Chen *et al.,* 2017** and **Radoshevich and Cossart, 2018)**.

The intracellular mobility and cell-to-cell

spread of *L. monocytogenes* require another surface protein, ActA (a protein encoded by *actA*), which is cotranscribed with PC-PLC and mediates the formation of polarized actin tails that propel the bacteria toward the cytoplasmic membrane. At the membrane, bacteria become enveloped in filopodium-like structures that are engulfed by adjacent cells, resulting in the formation of secondary double-membrane vacuoles. Subsequent lysis of the secondary double-membrane vacuoles signals the beginning of a new infection cycle, which is dependent on PC -PLC upon activation by Mpl (a metalloprotease encoded by *mpl*. In this manner, *L. monocytogenes* replicates and spread within the host avoiding the extracellular space and evading the immune system **(de las Heras *et al.,* 2011** and **Muchaamba *et al.,* 2019)**.

Molecular methods, especially, polymerase chain reaction (PCR), were as diagnostic tool for epidemiological investigation **(Steckler *et al.,* 2018)**. In the present study, PCR was successfully amplified virulence genes of *hly*A, *int*A, *int*B, and *prf*A in the 5 isolates diagnosed bacteriologically as *L. monocytogenes* at respective specific base pair 174, 343, 800 and 1052 respectively as shown in Figures 1 2 ,and 3.

The results revealed that, all isolates positive for the *Int*B and *Inl*A genes as showed in Photoograph 1 and 2. *Inl*B is a protein located on the same operon as *inl*A and they are a surface proteins required for internalization of *L. monocytogenes* into host epithelial cells, such as macrophages, fibroblasts, hepatocytes, epithelial and endothelial cells **(Su *et al.,* 2016)**. It has been reported that 96% of clinical strains of *L. monocytogenes* express full-length *InlAB* operon as compared to 65% food-associated strains **(Su *et al.,* 2016)**.

Additionally, and as shown in Photo, 3 all isolates were positive for the *hly*A, which encode listeriolysin O, and highly species specific and supplied easily *L. monocytogenes* differentiation from other hemolytic *Listeria* spp. **Deneer and Boychuk (1991)** mentioned that, Listeriolysin O (LLO) is a 60-kDa protein; as Listeria is engulfed by the host cell, they are enclosed within an intracellular vacuole that is surrounded by a membrane. LLO is a pore-forming toxin, essential for lysing the vacuolar membrane in the host cell, thus facilitating the escape of *L. monocytogenes* from the vacuole.

*Prf*A is the only regulator identified to date in *Listeria* spp. which is directly involved in the control of virulence gene expression. This protein is the main switch of a regulon including the majority of the known listeria virulence genes. Some of the members of the regulon, including LIPI-1 genes (with *prf*A itself) and several genes of the subfamily of secreted internalins (e.g., *inl*C of *L. monocytogenes* and i-*inl*E of *L. ivanovii*), are tighly regulated by *Prf*A; others, such as the *inl*AB operon, are only partially regulated by *Prf*A **(Nadon *et al.,* 2002)**. However, the expression of some listerial virulence genes is totally independent of *Prf*A, as is the case for the SMase gene of *L. ivanovii*, smcL, and the *inl*GHE internalin locus of *L. monocytogenes*. There is evidence that *Prf*A also negatively regulates some *L. monocytogenes* genes, such as the stress response mediator gene *clp*C and the motility-associated genes *mot*A and *flaA* **(Ollinger *et al.,* 2009)**. This suggests that *Prf*A has a more global regulatory role in *L. monocytogenes*.
To sum up, the positively of *L. monocytogenes* isolates for the four tested genes indicates that these isolates possess the properties of virulent strain and their sequences may be further investigated to explore the differences between pathogenic and less pathogenic strains. Our molecular results agree with that recorded by **Gaber*et al.,* 2014** and **Osman *et al.,* 2016**. On the other hand, **Gerstel *et al.* (1996)** showed that, none of other *Listeria* spp. than *L. monocytogenes* isolates amplified these tested virulence genes, which can be explained as the genetic information of these genes in other *Listeria* spp. could be divergent from that of *L. monocytogenes.*

Due to difference in the strains and techniques used for extraction of proteins, the obtained results could not be accurately compared with results of similar previous studies especially for the band sizes. It is known that the catabolism of added sugars might change the pH of

the medium, resulting in the suppression of some proteins and enhancement of others **(Datta and Kothary, 1993)**. Likewise, temperature and growth phase are important factors that influence the expression of many virulence factors, such as internalin, listeriolysin, phospholipases and actin polymerization proteins **(Dramsi *et al.*, 1993)**.

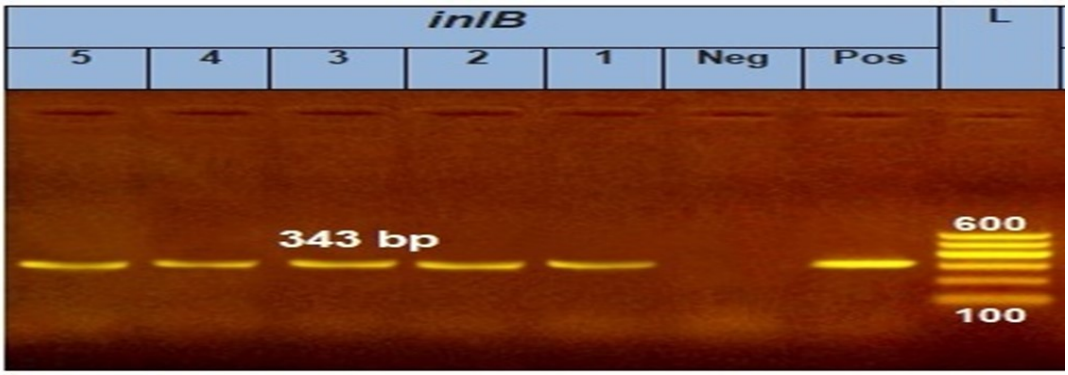

Photo. (1): Detectionof *inlB* gene among *L. monocytogenes* using polymerase chain reaction (PCR).**L**: DNA ladder (100 - 600 bp.). **Pos.:** Positive control. **Neg.:** Negative control. **Lanes (1-5):** showing positive *inlB* gene.

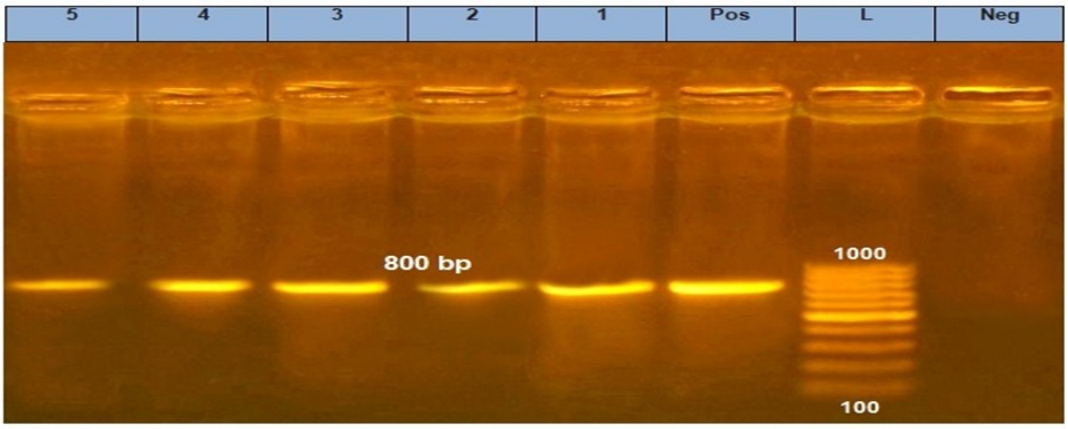

Photo. (2): Detection of *inlA* gene among *L.monocytogenes* isolates using polymerase chain reaction (PCR).
L: DNA ladder (100 -1000 bp.). **Pos:** Positive control. **Neg:** Negative control. **Lanes (1-5):** showing positive *inlA* gene.

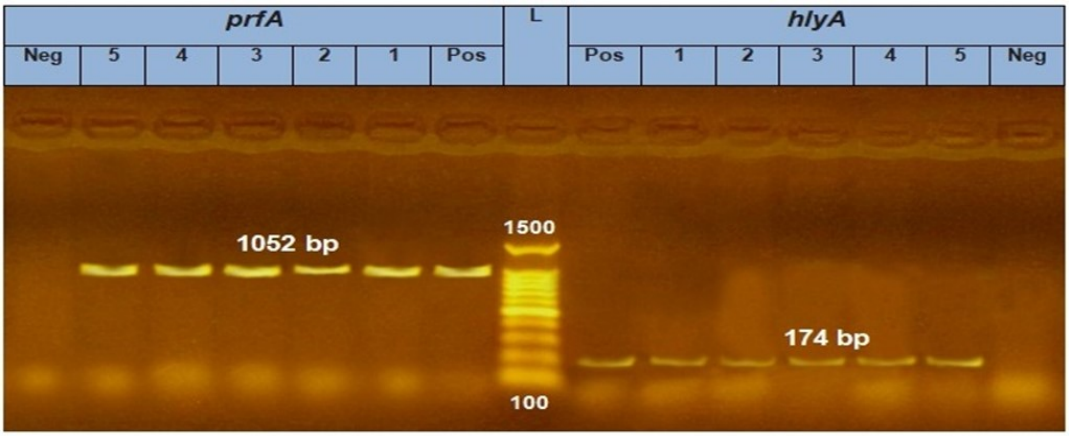

Photo. (3): Detectionof *prfA* and *hlyA* genes among *L.monocytogenes* isolates using polymerase chain reaction (PCR). **L:** DNA ladder (100-1500bp.), **Pos:** Positive control. **Neg**: Negative control. **Lanes (1-5)**: showing positive *prfA* and *hlyA* genes.

Conclusion: *L. monocytogenes* is an intracellular opportunistic organism found as contaminants of environment and different materials. It can multiply at a higher rate in poorly stored silage and rotting vegetation in which these are aerobic conditions and a pH that is higher than 5.4. Also it can survive at a refrigerator temperature, so that; *Listeria* infection is most prevalent during winter. These contribute as a source of infection to animals. The source of infection was most probably feeding on low quality grass silage. On the basis of the obtained data, we concluded that, *L. monocytogenes* is susceptible to most commonly used antimicrobial drugs. PCR results revealed that, all isolates of *L. monocytogenes* were positive for the presence of *inlA, inlB, prfA* and *hlyA* genes, this indicates that, these isolates possess the properties of virulent strain and their sequences may be further investigated to explore the differences between pathogenic and less pathogenic strains that leading to variety in clinical picture of the disease. Based on the above conclusions, the following recommendations are forwarded.

- As *L. monocytogenes* does not multiply to any month. Significant extent in effectively preserved silage, which is characterized by anaerobic storage, high concentration of organic acids and a pH below 4.5, the silage should be stored under these characterized conditions to maintain its quality.
- Incorporation of silage into the diet should be gradual and provision of green pasture should be encouraged.
- Spread hay out and avoid or lower overhead feeders if possible
- Susceptible animals' specially young and pregnant one should not be exposed to wet, cool, unhygienic environment and not feed on silage.
- Silage that is obviously decayed should be avoided.

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