# Overview on *Listeria monocytogenes* ecological niches, antibacterial susceptibility and virulence in bovine, caprine and ovine udder infection as a public health implication.

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## 1. Abstract

A total of 410 milk samples were collected from different ruminant species as the following ; cows (n = 110), buffaloes (n = 87) ) , does (n=108) ) and ewes (n=105) from different Egyptian farms .The results revealed that prominence of *L.monocytogenes* in the examined milk samples are 1.72 % at apparently healthy cow , 2.77 % in subclinical mastitic cow and in apparently healthy goat is 1.38 % and this has a public health importance. The recovered isolates identified biochemically as *L.monocytogenes* confirmed phenotypically by positive hemolytic reaction and motility , followed by molecular confirmation using specific primer sequences of 16s *rRNA* at 1200 bp followed with sequencing and accession on genbank . Antibiogram of the isolated *L. monocytogenes* are demonstrated resistance to tetracyclines, lincomycin, streptomycin, cefotaxime, Trimethoprime sulfamethoxazole, florphenicols, and clindamycin, while adherence assay for test glass tube polystyrene microtiter plates conclude that *L. monocytogenes* was able to form a strong biofilm.

**Key words:** *L.monocytogenes*, mastitis, ruminant, sequencing, virulence, antibacterial, public health

## 2. Introduction

*Listeria monocytogenes* is ubiquitous bacteria. It causes listeriosis, a serious infectious disease which occurs as consequence of consumption of food contaminated with this pathogen bacterium. The frequency of incidence of listeriosis is low (1%), but with high mortality rate (30%) [1]. *Listeria monocytogenes is* Gram-positive, facultative intracellular pathogen, rod-shaped flagellate, non-spore forming and catalase-positive bacterium which is responsible for causing severe foodborne infections and serious disease in different animal species as 'cattle, sheep, and goats' [2 , 3]. Listeriosis is caused by members of the genus Listeria, *L. monocytogenes* is the primary pathogen in humans and animals, [4] causing severe lesions as septicemia, meningitis, encephalitis and abortion, and also it is transmitted by eating food and water contaminated with pathogen. *Listeria monocytogenes* can be found in moist environment conditions as low temperatures, pH, high salt concentrations and multiplication in refrigerated temperatures, so it become as a serious to public health in different foods processed such as cheeses and dairy products [5], milk and milk productions [6], so food intake and direct transmission between humans and animals represents listeriosis [7].

Mastitis remains a major challenge to the worldwide dairy industry which causes inflammation of the mammary gland of dairy animals with losses attributable to reduced milk production, discarded milk, early culling, veterinary services, and labor costs [8 , 9] and believed the main

economic problems influencing the dairy industry and threaten animal welfare, quality of milk, and public health [10] **.** *L. monocytogenes* is a temporally resident of the intestinal tract in humans, with 2–10% of the hosts, even though rare, the dairy products are contaminated by listeria can cause listeriosis and a serious illness[11] Unfortunately, the pasteurization of raw milk does not remove more over risks of dairy product contamination by *L. monocytogenes* [12]. Animals can become infected by the ingestion of contaminated food by *L.monocytogenes* as contaminated roughage, pathogen inhalation, sawdust bedding, and farm yard manure [13] therefore, distributing untreated manure for agricultural purposes is regarded as a primary risk factor for the transmission of *Listeria* spp.[14].Recently, studies showed the virulence strain of *L. monocytogenes* prevalent in Egypt, so The present study aimed to follow raw domestic ruminant milk related *L.monocytogenes* isolates , occurrence, cultural, biochemical and molecular characterization, pathogenicity, and antimicrobial susceptibility profiles were assessed.

## 3. Materials and Methods

### Sample collection

A Total of 410 milk samples were collected from cows ($n = 110$), buffaloes ($n = 87$), goats (n=108) and ewes (n=105) depending of California mastitis test [15]. Then samples were transported and refrigerated quickly till analyzed.

### Isolation and identification of *Listeria monocytogenes*

10 ml of the milk sample were added to 90 ml of half Fraser broth CCFA (Oxoid; CM0895B and SR0166E). Incubate for 24 hr. at 30 °C, then transferred 100µl of culture to 10 ml of Fraser broth (Oxoid; CM0895B and SR0156E), incubate for 48 hr. at 35 °C. A loopful of broth was streaked onto the surface of oxford agar media and incubated for 24 to 48 hr. at 37 °C. *Listeria* isolates were identified according to [16] by the Gram's stain and biochemical identification as follows; catalase, esculin, xylose, hemolysis, motility test).

### PCR and sequencing of *L. monocytogenes*

The QIAamp DNA Mini Kit (Qiagen, Germany, catalogue no. 51304) according to the manufacturer instructions used for DNA extraction. Master Mix used for PCR was Emerald Amp GT PCR Master Mix (Takara, BIO INC., Japan, and Code No. RR310A) by using oligonucleotide primers sequences 16s r RNA[17] . Table (3).

### Virulence traits of *Listeria monocytogenes*

The classical tests for *L.monocytogenes* virulence examined were the Anton's test (rabbits), Vero cells assay. An assessment of the adherence formation assay according to [18] through the tube method and plate assay, as described by [19].

### Antibacterial susceptibility test

All recovered pure isolates were examined by the disc diffusion method [20] subjected to a susceptibility list of antibiotics (Oxoid: Ampicillin (25 µg), Penicillin G (10IU), Amoxicillin/clavulanic acid (10 µg), Enrofloxacin (10 µg), Ciprofloxacin (5 µg), Amikacin (30 µg), Gentamicin (10 µg), Kanamycin (30 µg), Streptomycin (10 µg), floramphenicol (10 µg), Tetracycline (30 mg), Trimethoprim-sulfamethoxazole 1 : 19 (25 µg), Cefotaxime (30 µg), Lincomycin (2 µg), Clindamycin (2 µg), Erythromycin (15 µg), Rifamycin (5 µg ),Vancomycin (30 µg). Isolates were cultured in trypticase soy broth (TSB) supplemented with 0.6% yeast extract, and transferred to Mueller– Hinton agar (Oxoid). The plates were incubated at $37^0$C for 48 hours.

## 4. Results

The collected milk samples examined for surveying of mastitis using CMT and the results revealed that; Cows (n=110) described by CMT to apparently healthy 52.72% (n= 58/110), subclinical mastitic 32.72% (n = 36/110) and clinical mastitic 14.54% (n = 16,110). Buffaloes (n = 87) to

apparently healthy 79.31% (n = 69/87), subclinical mastitic 12.64% (n = 11/87), clinical mastitic ( n = 7) 8.04% (7/87) .Goats (n=108) ) apparently healthy 66.66%( n = 72/108) , subclinical mastitic 24.07% (n = 26/108), clinical mastitic ( n = 10/108) . Ewes (n=105) apparently healthy 75.23%( n = 79\105) , Subclinical mastitic 20 % ( n = 21/105), clinical mastitic 4.76%(n = 5\105) . *L.monocytogenes is* confirmed phenotypically by positive hemolytic reaction and motility , followed by molecular technique PCR with specific primer sequences and were accessed in genbank under the following criteria; BankIt2439314S1.EGY.COW.2019 MW751827,BankIt2439314S2.EGY.cow.2019 MW751828,BankIt2439314S3.EGY.goat. 2019 MW751829 .The incidence rate of *L.monocytogenes* in the examined milk samples was as follow; apparently healthy cow is 1.72% ( 1/58) , subclinical mastitic cow is 2.77 %(1/36) and apparently healthy goat is 1.38 %(1/38) and there is no evidence for *L.monocytogenes* in the other samples. The recovered isolates identified biochemically as *L.monocytogenes* confirmed phenotypically by positive hemolytic reaction and motility (table 1), followed by molecular technique PCR with specific primer sequences (fig 1), also the virulence traits as Vero cell assay and Anton's test confirmed that the recovered isolates were *L.monocytogenes* (table1) followed by studying the adherence ability to glass and plastic at which all isolates were strong adherent to plastic and glass except that become from apparent healthy cow milk was moderate adherent to glass (table 1).The Antimicrobials susceptibility of the isolated *L.monocytogenes* recorded in (table 2) at which the isolates were resistant for tetracyclines, lincomycin, streptomycin,cefotaxime,Trimethoprime,sulphamethaxole,florphenicols,and clindamycin.

**5. Discussion**

Mastitis considered as remarkable disease which affects dairy animals, that not only causes changes in glandular tissues but affects the quality and quantity of milk production , and also the health risk to consumers due to the presence of zoonotic pathogens and drug residues [21]. Subclinical mastitis can difficult to be detected because of a lack of clinical signs which easily identified by visual inspection and palpation of the udder compared with clinical mastitis, so reliable diagnostic methods are needed to detect subclinical mastitis such as CMT [22]. In the present study the examined milk samples subdivided as the following follow; cow milk (n=110) described by CMT to apparently healthy (n= 58/110) , subclinical mastitic ( n= 36/110) , and clinical mastitic ( n=16\110) ,buffalo milk (n = 87) ) to apparently healthy (n = 69/87), subclinical mastitic (n=11/87) , clinical mastitic( n =7/87) ,goat milk (n=108) ) apparently healthy (n = 72/108) , subclinical mastitic ( n =26/108), clinical mastitic( n =10/108) and ewe milk (n=105) ), apparently healthy (n = 79/105) , subclinical mastitic ( n = 21/105), clinical mastitic( n= 5/105) .The Result of the current study proved the occurrence of *L.monocytogenes* at the examined milk samples were as the following; apparently healthy cow milk is 1.72% (1/58) , subclinical mastitic cow milk is 2.77 %(1/36) and apparently healthy goat is 1.38 %(1/38) , while the other milk samples showed negative results for *L.monocytogenes*. The nearly similar results of *L. monocytogenes that* isolated from cow's milk samples were notified by [23] and [24], however the higher percentage was reported by others [25] and [26]; on the other hand, the higher frequencies of *L.monocytogenes* that isolated from goat and sheep's milk samples were obtained by [24], [25]; [27] and [26]. The higher prevalence of *L.monocytogenes* in buffaloes's milk samples were recorded by [24] , [25] and [23].The recovered isolates identified biochemically as *L.monocytogenes* confirmed phenotypically by positive hemolytic reaction and motility , followed by molecular technique PCR with

specific primer sequences and accessed in Genbank under the following criteria;BankIt2439314S1.EGY. COW.2019 MW751827, BankIt2439314 S2.EGY.cow.2019 MW751828,BankIt243 9314S3.EGY.goat.2019 MW751829.
Between animals, listeriosis can affect sheep and goats and the pathology may extend in different clinical forms and include mastitis.

The excretion of *Listeria* in milk can preserve throughout lactation and contribute to an increased risk of milk product contamination [28]. *L. monocytogenes* isolated from milk of subclinical mastitis sheep which characterized by a persistent shedding of Listeria and an apparently normal milk consistency and appearance [29].The results of adherence assay to test glass tube assessed by 0.1% Crystal Violet stain showed that two isolates of *L. monocytogenes* were able to strongly form a biofilm on a glass surface, while one isolate from cow apparent healthy was moderately adherent, also the three isolates of *L. monocytogenes* were screened for their adherence to polystyrene 96-well microtiter plates at different degrees; the results concluded *L. monocytogenes* was able to form a strong biofilm on polystyrene ($OD570 \geq 1$) .

The microbial community on the teat surface are varied from one farm to another according to many different factors as microbial load , type in the bedding material and milking machines that can contaminate the surface of teat and can potentially enter to the milk. This circumstance focuses on the requirement for successful cleanliness hindrances since control of microbial impurity before treating e.g. from animals having inflammation of udder tissues, the nature or water, together with process control have been distinguished as safe elements for creating safe milk. The propensity of *L. monocytogenes* for infecting and propagating in food and on other non-nutritive surfaces is related to its ability to form biofilms and to biotransfer; both these characteristics confer adhesive property and grant protection to the microorganism. In the current study the antibacterial susceptibility test for the recovered isolates revealed that *L. monocytogenes* displayed resistance for five antibiotics: tetracyclines, lincomycin, streptomycin, cefotaxime and clindamycin. The biofilms thus formed are viable for months or even years; this allows for the occurrence of recurrent contamination of food (xxx). Certain additional characteristics of *L. monocytogenes* such as its ability to multiply in the presence of environmental acids, cold refrigerated conditions, high concentrations of sodium chloride and other conditions that are usually averse to growth of other pathogenic bacteria, further aid in the propagation and perpetuation of the contamination [30] . Raw drinking milk (RDM) has a diverse microbial flora which can include pathogens transmissible to humans. The main microbiological hazards associated with RDM from cows, sheep and goats [27]. Although several animal-derived *L. monocytogenes*-contaminated food products, including raw milk, pasteurized milk, chocolate milk, butter, soft cheeses, and processed meat and poultry products, have been implicated as sources of human listeriosis cases and outbreaks [2 ,31 ,32 ,33 ,34 ].

## 6. Conclusion

The present study showed evidence of the contamination leading to the presence of *L. monocytogenes* in raw domestic ruminant milk and also this study is confirmed a strong in vitro ability for adherence formation and pathogenic capability of *L. monocytogenes* if discovered in the milk, so in order to reduce the exposure, hygienic milking conditions must be used for the milk to avoid public health hazard.

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Table (1): Virulence trait of recovered *L.monocytogenes*

| Item | *L.monocytogenes* | | |
|---|---|---|---|
| Origin | apparently healthy cow | subclinical mastitic cow | apparently healthy goat |
| Motility at 25 °c | + | + | + |
| Hemolytic activity | + | + | + |
| Anton's test | + | + | + |
| Vero cell | Cell deformation | Cell deformation | Cell deformation |
| Glass adherence | Moderate | Strong | Strong |
| Plastic adherence | OD= 0.232 | OD= 0.345 | OD= 0.324 |

Table (2): Antibiotic susceptibility of the recovered *L.monocytogenes*

| Antimicrobials | *L.monocytogenes* | | |
|---|---|---|---|
| | apparently healthy cow | subclinical mastitic cow | apparently healthy goat |
| Ampicillin 25 µg | S | S | S |
| Penicillin G 10IU | S | S | S |
| Amoxicillin/clav. 10 µg | S | S | S |
| Enrofloxacin 10 µg | S | S | S |
| Ciprofloxacin 5 µg | S | S | S |
| Amikacin 30 µg | S | S | S |
| Gentamicin 10 µg | S | S | S |
| Kanamycin 30 µg | S | S | S |
| Streptomycin 10 µg | R | R | R |
| Floramphenicol 10 µg | R | R | R |
| Tetracycline 30 mg | R | R | R |
| Cefotaxime 30 µg | R | R | R |
| Lincomycin 2 µg | R | R | R |
| Clindamycin 2 µg | R | R | R |
| Erythromycin 15 µg | S | S | S |
| Rifamycin5 µg | S | S | S |
| Vancomycin 30 µg | S | S | S |
| Sulfamethoxazole- 25 µg | R | R | R |

Table (3): Primers for PCR detection

| | | |
|---|---|---|
| primer | GGA CCG GGG CTA ATA CCG AAT GAT AA, | Amplified product |
| 16s rRNA | TTC ATG TAG GCG AGT TGC AGC CTA | 1200bp |

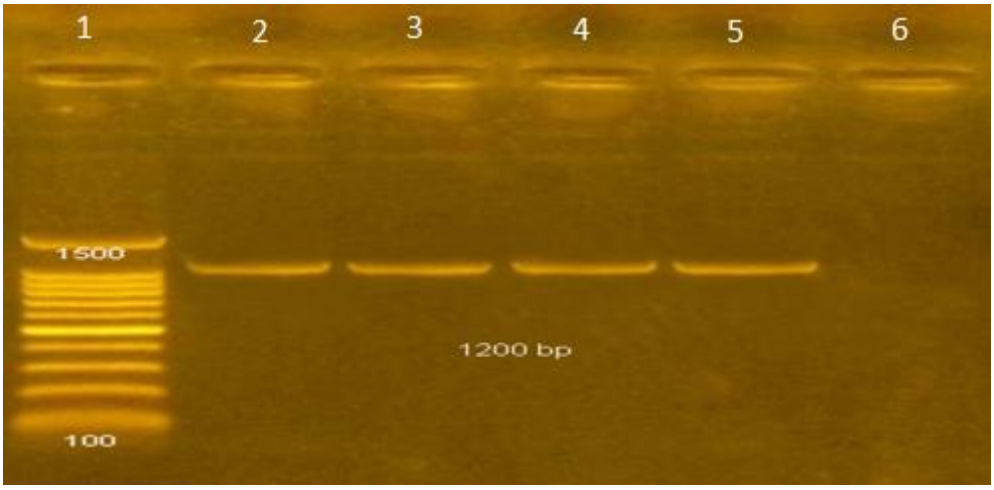


Fig (1): Agarose gel electrophoresis showing positive amplification at 1200 bp. for 16s *rRNA* of *L. monocytogenes* by using specific primer sequences "L1: 1500 bp. DNA ladder , L2- control positive ATCC 19115, L3: *L .monocytogenes* from Apparently healthy cow, L4: *L. monocytogenes* from subclinical mastitic cow, L5: *L. monocytogenes* from Apparently healthy goat, L6: control negative" .