Assiut Veterinary Medical Journal **Assiut Vet. Med. J. Vol. 70 No. 181 April 2024, 232-243**

Assiut University web-site: www.aun.edu.eg

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM RAW MILK IN ASSIUT CITY

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Received: 13 January 2024; **Accepted:** 31 March 2024

ABSTRACT

Among a food-associated pathogen, Listeria monocytogenes has become the most important one. It can cause a "high fatality rates 20–30%" in comprasion to another foodborne bacteria which causing dangerous disease called listeriosis. Listeria outbreaks are often linked to dairy products and milk. This article intends to review phenotypic and genotypic characters of *L. monocytogenes* in Assiut city. How common *L. monocytogenes* is determined in different sources of raw milk (20 market milk, 40 buffleo milk and 40 cow milk). By detection of 3 virulence genes and its sequencing (*hly*A, *inl*B and (*prf*A) can deterimined the pathogenic potential of the isolates. Firstly enrichment samples in fraser broth, then, plating on to ALOA®, Merck. Finally a multiplex-PCR,was used for identification of suspected colonies. The results revealed that 8 (40%), 6 (15%) and 11 (27%) of samples that collected from market, buffalo and cow raw milk were positive respectively. Antibacteerial sensitive test showing in vitro thai Listeria mmonocytogenes had a higher sensitivity to Sulfa methoxazoletrimthoprim (SXT) and Ciprofloxacin, (CIP) followed by Chloramphenicol (C), low sensitive to Gentamicin (CN), it resisted Erythrommycin (E) and Amoxicillin (AX). The PCR results for isolates have hly A Inl B and prf A genes of *L. monoctogenes*.

Ketwords: *raw milk PCR, L. monocytogenes hly A, Inl B and prf A.genes*

INTRODUCTION

Renato *et al.* (2015) showed that *L. monocytogenes* was causing listeriosis disease that affects human being and ruminant. (Ikeh, *et al.,* 2010; Khan *et al.,* 2013) recorded that spreading of *L. mmonocytogenes* infestion as a result of various types of microorganisms cauasing food poisoning, that exist in milk and milk products.

Todar, (2009) postulated that eating of any food contamminted with *L.* monocytogenes a serious disease infected humans and animals (Listeriosis) were occuried, in nine ninety% of cases and rarely from the environment. The infective dose of *L. monocytogenes* is not precisely determined. Ooi and Lorber, (2005) showed the value of hundrud/thouand *L. moncytogenes* per gram

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of foodstuff cause listeriosis in humans according to most of researchers. EFSA ECDC, (2015) indicated that immunocompromised individuals, pregnant women, neonates, and the old aged person which acted as the higher risk group for infection with *Listeria monocytogenes*. Gray *et al.* (2004) studied that infection with listeria monocyogenes is equally in both animals and human. The risk of infection with listeria related with food is high due to many reasons as its wide variety of reservoirs (Lianoe and Sofos 2007) it was able to colonize abiotic surface), (Poimenidou *et al.,* 2016b) and to withstand environmental stresses (Poimenidon *et al.,* 2016a) mmicroorganimm can able to find in food plants for several months or years. O'Connor *et al.* (2010) noticed that there are varsious ways for contaimmanation of food with *Listeria* and transimmmmted to man.The most common clinical symptoms as a result of infection with listeria were meningitis,septicemmia, and the mmost infected person were children, immune compromised patients and pregnant (Şanlıbaba and Tezel, 2018). Whereas, in economically important animals, the disease by *listeria* causing abortion and central nervous system diseases in cattle and sheep, in livestock industry and can lead to decreasing of milk yield and high mortality rate in breeding farms. Skin and intestinal tract of livestock animals acted as habitats for many foodborne microorganism and opportunistic bacteria which contaminated milk during milking.

Theivagt *et al.* (2006); Vera *et al.* (2013) and U.S. Centers for Diseases Control and Prevention Listeria 2022) showed that phenotypically *Listeria mmonocytogenes* is a negative Gram stain rod-shaped, has no capsule has no spore 0.5 mm iin width and 1-1.5 mmm in lengthened forming sigle short chain. Moreover Robinson *et al.* (2000) revealed that taxonomically, *Listeria mmonocytogenes* is classified into 6 species. *L. monocytogenes, L.ivanovii, L. seeligerol, L. innocua, L.welshimer from those only L. monocytogenes and L. ivanovii* and *L. graii)a*re pathogenic Although it was be infect both human and animals, L. ivanovii is a principally an animal pathogen that rarely occurs in man .

Genotypic characterization of *L. monocytogenes* depend on detection of virulence genes of bacteria or the products of gene. *Listeriolysin O (LLO)* as a marker is required for intracellular survival of invading bacteria in mammalian host. *Hly*-A. *L. monocytogenes* is the gene encoding LLO can be produce other virulence proteins in side of LLO as PI-PLC and PC- Ward *et al.* (2010).

(Halberg- Larasen *et al.,* 2014) detected that spreading of *L. monocytogenes* infection be related to drinking of milk and causing high mortality rate 30% and economic problems in the dairy industry.

Reissbrodt, (2004) documented that in conventional methods the detection of L. monocytogenes from foods samples requires the use of enrichment cultures then followed by selective plating. Reissbrodt, (2004) and Gasanov *et al.* (2005) recorded that a significant growth must be occurred not only in selective culture and enrichment procedures, but also using of various new and rapid detection methods depond on antibody and antigens reaction. PCR has been used for rapid, sensitive and specific diagnosis of foodborne micro-organisms (Olsen *et al.,* 1995).

MATERIALS AND METHODS

1- Products tested:

One hundred raw milk samples collected randomly from different locations in Assuit City including (market, buffulo and cow milk 20, 40 and 40 samples, respectively). The samples were transported into sterile plastic bags and transported in an ice box to the laboratory .

2- Microbiological analysis for Listeria isolation:
As described by Becker *et al.* (2006) for isolation and identification of L. monocytogenes in this study, ISO 11290 method was used.

2-1-Preenrichment: (Fraser and Sperber, (1988)
The first enrichment culture, 25 g of samples to 225 ml of half Fraser broth (Merck) in stomacher bag and were homogenized by a stomacher (Lab blender 400, Seward Medical, London, UK) and incubated for 24 h at 30 °C .

2-2-Selective enrichment:
The second enrichment culture 0.1 ml of half Fraser broth to 10 ml of Fraser broth were added and incubated at 37 °C for 48 h in aerobic conditions.

2-3-Isolation and identification:
2-3-1 Isolation
A loopful of enriched Fraser broth-culture was streaked on the agar plate ALOA®, Merck agar (Merck) and OXFORD agar for selective plating (the tubes that present a blackening) was performed. The Fraser broth-cultures were incubated for 24 h at 37 °C. On tryptic soy agar with 0.6% yeast extract (Oxoid, Basingstoke, UK) three to five colonies were re-streaked.

2-3-2- Identification
According to FDA bacteriological Analytical Manual (Hitchins, 1995). Colonies were confirmed by catalase, oxidase, methyl-red, voges proskauer and urease biochemical tests. Also, sugar fermentation as (rhamnose, xylose and mannitol) were confirmed. On CAMP test and blood agar, the isolates were further performed by hemolysis according, to the manufacturer's recommendations selected colonies, initially identified as Listeria spp. and transportd to 5% sheep blood with Columbia Agar (bioMérieux). Finally, by using the PCR, we can determine the hemolysis type and identification was performed.

2.!.Antimicrobial sensitivity tests in –Vitro:
Against different antibiotics, *L. monocytogenes* strains were subjected and the antibiotic discs are Erythromycin (E), Ceftriaxone (CRO), Gentamycin (CN), Clindamycin (DA), Amoxicillin (AX), Ciprofloxacin (CIP), Chloramphenicol (C) Sulfamethoxazole-trimthoprim (SXT) Ciprofloxacin) CIP) by using the disc diffusion method (NCClS, 1999) multiplex-PCR assay. 2.5. Genotypic detection of isolated *L.monocytogenes* and and some virulence in them by using of Polymerase chain reaction (PCR)

Three sets of primers were used for detection of virulence genes in *Listeria monocytogenes* strains (16 rRNA; internalin heamolysin (*hly*A); internalin B (*inl*B) and positive regulatory factor A (*prf*A).

DNA Extraction (Vera *et al.,* 2013)
By using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). DNA was extracting. 200 µl of the sample suspension with 20 µl of proteinase K and 200 µl of lysis buffer was incubated at 56OC for 10 min. To the lysate after incubation, 200 µl of 100% ethanol was added then washing the sample and centrifuged following the manufacturer's recommendations. 100 µl of elution buffer provided in the kit was eluted with Nucleic acid.
For DNA amplification reaction of L.monocytogenes we can be using QIA kit was amp used kit (Wehlan and &Kreft 2001).).

PCR amplification
The reaction was performed in an Applied biosystem 2720 thermal cycler, in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan) primers were utilized. 5.5 µl of water, 1 µl of each primer of 20 pmol concentration and 5 µl of DNA template .

3.-Analysis of the PCR Products.

1. By electrophoresis the products of PCR were separated. On 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm.

2. A Gelpilot 100 bp plus Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. For gel analysis twenty µl of the products was loaded in each gel slot .

3. By a gel documentation system (Alpha Innotech, Biometra). The gel was photographed.Through computer software the data was analyzed .

Oligonucleotide Primer. From Metabion (Germany) are listed in table (2,3) primers were supplied.

Table A: amplicon sizes

Ampliconation (35 cycles		Amplified segment (b p)	Primary denaturation	Reference
Secondary denaturation	Annealing			
94C 30sec.	60C 1 min	1200	94˚C 5 min	Kumar *et al.*, 2015

Target agent	Target genes	Final extension	Primers sequences
Listeria mmonocytogenes	16 S rRNA	72˚C 12 mmin	GgACCggggCTA ATA CCg AA TgATAA TTC ATg TAggCg AgT TgCAgCCTA

Table B: Target genes, Primers sequences and Final extension

Table C: During cPCR cycling conditions of different primers4-

Genes	Primmary denaturation	Secodary denaturation		Annealing	Extension		No. of cycles	Final extension	
prfA	. 94˚C 5 min.	94˚C	30 sec.	50˚C 50 sec	72˚C	1 min	35	72C	10 min.
inlB			5min.	55C 40 sec.		.			
hlyA			30sec.	50C 30 sec.		72˚C 30 sec.			7min.

Table D: Sequences sources oligonucleotide and primmers.

Primer	Sequence	Amplified product	Reference
hlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA TGT-CAC-TGC-ATC-TCC-GTG-GT CCYTTTTATGTACCCAYGA	174bp	Hitchins, 1995
inlB	CTGGAAAGTTTGTATTTGGGAAA TTTCATAATCGCCATCATCACT	343 bp	

prfA	TCT-CCG-AGC-AAC-CTC-AAC-CTC-GGA-ASS TGG-ATT-GAC-AAA-ATG-GAA-CA	1052 bp	Sambrook, et al.,1989

Statistical analysis

By using Chi-square analysis. All statistical analysis the relationship between the true/false positive results and the food categories were analyzed using Chi-square analysis.

RESULTS

Types of Samples	No of examined samples	The positive isolates	isolates percentage %.
Market milk	20	8	40
Buffalo milk	40	6	15
Cow milk	40	11	27.5
Total	100	25	82.5

Table 1: Preverance of *l, monocytogenes* in the Market, Buffalo and Cow milk according to conventional method.

Table 2: In-vitro antimicrobial sensitivity test for isolated L.monocytogenes .

	sxt	CN	E	CRO-	NV	D	AX	CIP	C
Market milk									
% S	83.3	16.7	Zero	33.3	Zero	33.3	Zero	83.3	Zero
% R	16.7	66.7	100	50	83.3	66.7	100	Zero	33.3
% I	Zero	16.7	Zero	16.7	16.7	Zero	Zero	16.7	66.7
Buffulo milk									
% S	100	33.3	Zero	33.3	Zero	66.7	Zero	66.7	33.3
% R	Zero	Zero	100	Zero	100	Zero	100	Zero	33.3
% I	Zero	66.7	Zero	66.7	Zero	33.3	Zero	33.3	33.3
Cow milk									
% S	70	30	10	50	zero	20	zero	90	80
% R	20	40	90	10	90	40	100	Zero	10
% I	10	30	zero	40	10	40	zero	10	10

% S sensitive
% R Resistance
% I Intermediated

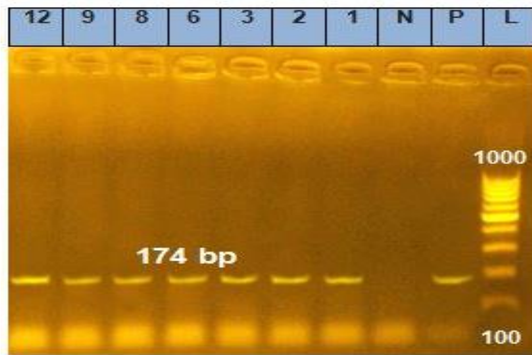

Photo 1: hylA gene.
Lane L: 100-1000 bp Ladder
Neg: Negative control
Pos: Positive controlat 174bp
Lanes 1 to 12: *L.monoytogenes (hyl* A positive

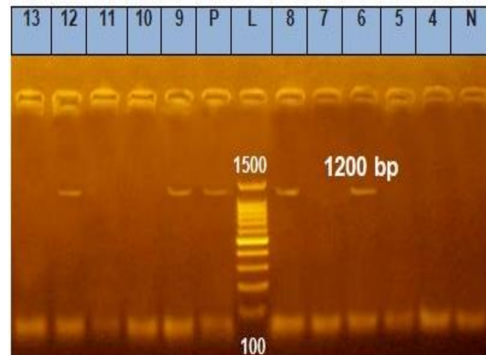

Photo 2: inlB genes.
Lane L: 100-1500 bp Ladder.
Neg: Negative control
Pos: Positive controlat 1200bp
Lane 1 to 12 :*L.monocytogenes in I* B

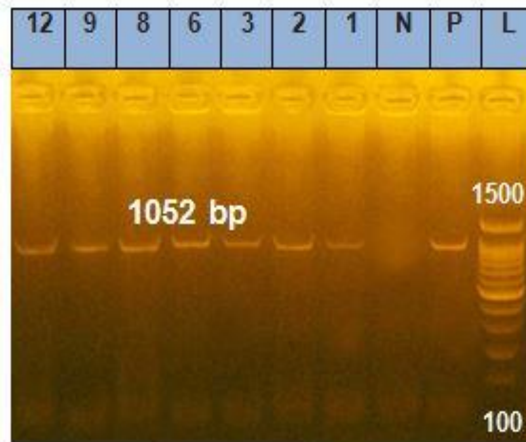

Photo 3: *prf*A genes
Lane L: 100: 1500 bp Ladder.
Neg: Negative control
Pos: Positive controlat 1052 bp
Lane 1 to 12: *L. monocytogenes prf* A

DISCUSSION

Adzitey *et al.* (2013): Liao *et al.* (2021) demonstrated that among over 200 foodborne dieases which transmitted by food acted as a growing health problem in all world. As a result of high mortality rates saucing by listeria infection and its ability to grow in refrigerator temperature. *L. monocytogenes* has special significance among foodborne pathogens (Lida *et al.,* 2014 and Anonymous, 2021).

In medical and veterinary medicine, *L. monocytogenes* is an important bacteria and can cause abortion, encephalitis in sheep and cattle and in other mammals, birds and fish cusing a variety of diseases (Kalrey *et al.,* 2008)..

Eating of fast food was the most human listeriosis cases (Kells and Gilmour, 2004) indicated that using of fermented dairy products in feeding that made of raw milk that contaimenated with *L. monocytogenes* either from environmental sources or postprocessing not directly related to raw milk or use of raw milk contaimented with *L. monocytogenes*.

Control of this bacterium during food processing is extremely difficult because of listeria is more spreading in food industrial environment and farm (Kells and Gilmour, 2004 and Sarfraz *et al.,* 2017). The ability of *L. monocytogenes* for causing lesions depened on the expression of virulence factors and immune status of patients those the most exposed to infection of *L. monocytogenes* Patients have weakened cell-mediated (Lecuit *et al.,* 2004).

In Table 1 the result showed that *L. monocytogenes* was isolated in 8,6 and 11 in market, bufullo and cow raw milk respectively. These percentages were (0.4, 150 and 0.275%) in market, bufullo and cow raw milk, respectively, which not agreed with that represented by (Zeinali *et al.,* 2017)

In Table 2, *L.monocytogenes* were sensitive to Sulfamethoxazole-trimthoprim (SXT) and Ciprofloxacin (CIP) followed by Chloramphenoicol (C), less sensitivity to Gentamicin (CN). Wheras resistant to Erythromycin (E) and Amoxicllin (AX) as in-vitro antimicrobial sensitivity test. These results agreed with (Yao 2022). and didnot agree with (Altuntas *et al.,* 2012).

The result of virulence tests for isolated listeria recorded that all *L. monocytogenes* showed narrow zone of, β-heamolysis in blood agar, and positive to CAMMP test that results were similar to that obtained by (Marrouf *et al.,* 2007 and and Rahimi *et al.,* 2020). Which can determine the pathogenicity (Maarouf *et al.,* 2007).

Wile, did not agree with that reported by (Yao 2022). *L. monocytogenes* PCR results were not detected in all isolated recorded that (16rRNA; *hly*A, *inl* B and *perf*A genes). with agreed with those recorded by (Ciolacu *et al.,* 2015) showing that existing of16rRNA genes in *L. monocytogenes* isolates, results recorded that, amplification was in all tested isolates.. while do not agreed with (Shen *et al.,* 2000). PCR results for: *inl* B gene in *L. monocytogenes* recorded that, *inL*B gene do not amplified in all tested isolates.

Similar data wererecorded by (Gelbicova and Karpiskova, 2012; Khen *et al.,* 2014; Ciolacu *et al.,* 2015 and Self *et al.,* 2019) result of PCR for *hly*A genein *L. monocytogenes* reported that *hly*Awas amplified in all tested isolates making products of 174bp .

It was not similar to (Gelbicova and Karpiskova, 2012 and Ciolacu *et al.,* 2015).the result of PCR of positive regulatory factor gene (*prf*A) in *L.monocytogenes* showed that, *prf*A gene wasn't amplified in all tested isolates .

In the present study, Table 1 by using conventional method postulated that *L.monocytogenes* was detected in raw milk samples7.5%, almost similar results by Aygun and Pehlivanlarmm (2006) recorded *L. monocytogenes* in 5% of milk samples , wheras, (Borucki, *et al.,* 2005) recorded that in 30 samples of sheep,goats and cow milk samples, wheras, Kasalica and Oljačić (2007) recored that in 30 samples of sheep, goat and cow milk the presence of *L.monocytogenes* was not established and (Mohamed, 2010) reported that *L. monocytogenes* was present in 1.7 and 3.3% in raw milk samples in farm a and b. also, Cabanes *et al.,* 2005) showed that listeria monocytogenes was present in 4% of raw milk samples, dairy products associated with *L. monocytogenes* infection. Cow milk is acted as carrier of the fatal listeriosisas. (Farber and Peterkin, (1991) and Sepahvand *et al.* (2022) .

Clinical healthy animals are represented as a carriers of *L. monocytogenes* and acted as a source of contamination of milk. The most common route of transmission of *L.monocytogenes*, is mainly milk due to unhealthy animals on the farm. listeria monocytogenes was isolated in 60% samples as result of poor quality of

prepared silage according to some literature data, (Vilar *et al.,* 2007). *L.monocytogenes* was isolated in 1% samples of milk collected from cows fed silage (Vilar *et al.,* 2007). Poor quality of prepared silage is the main source infection of animals by *L. monocytogenes*.

L. monocytogenes in food from reports speak of the presence of microorganism in food obtained in developed countries (Melanie and Siegfried, 2001; Karakolev, 2009)recorded that in Europe milk can be contaminated with *L. monocytogenes* with 2,5- 6 % of samples that indicating potential risk for human population from dairy products manufactured from milk according to microbiological studies (Donnelly, 2004).

L. monocytogenes acted as an exciting models of host–pathogen interaction at the cellular and molecular levels, as a result of various molecular virulence determinants have been a role in the cellular infection by *L. monocytogenes*. These virulence determinants include, among others, the internalins, listeriolysin O (LLO), *Act*A protein, two phospholipases, a metalloprotease, Vip protein, a bile exclusion system (BilE) and a bile salt hydrolase (Cabanes *et al.,* 2005; Cossart and Toledo-Arana, 2008; Sleator *et al.,* 2005). Targeting the hly gene, has been found to be a sensitive and rapid technique for confirmation of the identification of suspected *L. monocytogenes* isolated on selective/differential agar plates according to Polymerase Chain Reaction, results (Gouws and Liedemann, 2005)..

CONCLUSION

Finally, it can conclude that *L. monocytogenes* is the bacterium which can polluted raw milk causing disease called listeriosis. *L. monocytogenes* isolates were sensitive to Sulfamethoxazole-trimthoprim (SXT) and Ciprofloxacin (CIP) followed by Chloramphenoicol (C), less sensitive to Gentamicin (CN). Wheras, resistant to Erythromycin (E) and Amoxicllin (AX).

L. monocytogenes isolates were Positive for CAMP also it made β-zone of hemolysis. PCR result recorded that isolates are *L. mmonocytogenes* and have *hly*A, *inl*B and *prf*A virulence genes.

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الشكل الظاهري والجينى للستيريا مونوسيتوجين المعزولة من اللبن الخام في مدينة أسيوط

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أصبحت الليستريا المستوحدة ذات أهمية متزايدة كعامل ممرض مرتبط بالغذاء. يمكن أن يسبب مرضًا نادرًا ولكنه خطير يسمى الليستريات مع معدلات وفيات عالية (٢٠-٣٠٪) مقارنة بمسببات الأمراض الميكروبية الأخرى المنقولة بالغذاء. غالبًا ما يرتبط تفشي الليستريات بالألبان و منتجاتها. تهدف هذه الدراسة إلى مراجعة النمط الظاهري والوراثي لليستريا المستوحدة المعزولة من البن الخام في مدينة أسيوط للسيطرة على الليستريات ، وكانت أهداف هذه الدراسة تحديد مدى انتشار L. monocytogenes المصدر المختلف للبن الخام (٢٠ لبن السوق ، ٤٠ لبن جاموسى و ٤٠ لبن بقري) وتقييم القدرة الممرضة للعزلات من خلال تحديد ثلاثة جينات مرتبطة بالضراوة (hlyA و inIB و prfA) وتسلسلها. تم تخصيب العينات أولاً باستخدام طريقة التخصيب في مرق فريزر ، تليها الانماء على ALOA® ،Merck أجار. للتحديد النهائي للمستعمرات المشتبه فيها ، تم استخدام طريقة اختبار البلمرة المتسلسل المتعدد. لتقدير حدوث ومستويات الليستريا النيابة ، من عدد ١٠٠ عينة عشوائية تم جمعها من مصادر مختلفة ، ٢٠ عينة معزولة من اللبن الخام في السوق ، ٤٠ من ألبان الجاموس الخام و ٤٠ من البان البقر الخام. ٨ فقط (٠,٤٠٠٪) و ٦ (٠,١٥٠٪) و ١١ (٠,٢٧٥٪) من العينات التي تم جمعها من لبن السوق والجاموس والأبقار الخام على التوالي ، يشتبه في أن تكون العزلات المشتبه بها والمكتشفة على أجار أكسفورد ألوا هي Listeria spp. أظهر اختبار الحساسية لمضادات الميكروبات أن مستخلصات L. monocytogenes المعزولة حساسة للسلفاميثوكسازول - تريمثوبريم (SXT) (وسيبروفلوكساسين (CIP) متبوعًا بالكلورامفينويكول (C) ، حساسية ضعيفة للجنتاميسين (CN)) ، بينما كانت مقاومة للإريثروميسين (). E) و Amoxicllin (AX).

أظهرت نتائج اختبارات سلالات الليستريا المعزولة أن الليستريا مونوسيتوجينز كانت سلالات خبيثة حيث كان بعضها إيجابيًا لاختبار CAMP. أظهرت نتائج تحليل تفاعل البلمرة المتسلسل (PCR) لعزلات الليستريا منطقة ضيقة من انحلال الدم β على أجار دم الأغنام.

تم تحديد التلوث بـ L. monocytogenes في اللبن الخام مع الأخذ في الاعتبار الخصوصية العالية والحساسية للمقايسة متعددة الإرسال PCR المستخدمة ، وهي الطريقة الأكثر دقة لتحديد L. monocytogene