Prevalence, pathogenic capability, virulence genes, biofilm formation, and antibiotic resistance of *Listeria* in goat and sheep milk confirms need of hygienic milking conditions

Kamelia M. Osman[1], Tara Rava Zolnikov[2], Ahmed Samir[1], Ahmed Orabi[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt, [2]North Dakota State University, Developmental Science, Fargo, ND, USA

Goat and sheep milk is consumed by human populations throughout the world; as a result, it has been proposed as an alternative, nutrient-rich milk to feed infants allergic to cow's milk. Unfortunately, potentially harmful bacteria have not been thoroughly tested in goat or sheep milk. *Listeria monocytogenes* is a harmful bacterium that causes adverse health effects if ingested by humans. The purpose of this study was to estimate the prevalence and characterize the phenotype, genotype, virulence factors, biofilm formation, and antibiopotential of *Listeria* isolated from the milk of goat and sheep. Udder milk samples were collected from 107 goats and 102 sheep and screened for mastitis using the California mastitis test (CMT). Samples were then examined for the presence of pathogenic *Listeria* spp; if detected, the isolation of pathogenic *Listeria* (*L. monocytogenes and Listeria ivanovii*) was completed using isolation and identification techniques recommended by the International Organization for Standards (ISO 11290-1, 1996), in addition to serological, *in vitro* and *in vivo* pathogenicity tests. The isolates were subjected to PCR assay for virulence associated genes *(hly*A, *plc*A, *act*A, and *iap*). Pathogenic *Listeria* spp. were isolated from 5.6% of goat and 3.9% sheep milk samples, with 33.3 and 25% of these selected samples respectively containing *L. monocytogenes.* The results of this study provide evidence of the low-likelihood of contamination leading to the presence of *L. monocytogenes* in raw goat and sheep milk; however, this study also confirmed a strong *in vitro* ability for biofilm formation and pathogenic capability of *L. monocytogenes* if discovered in the milk. *L. monocytogenes* may be present in goat and sheep milk and in order to reduce the exposure, hygienic milking conditions must be employed for the milk to be considered a safe alternative for human consumption.

Keywords: *Listeria* species, Goat, Ewe, Virulence genes, Biofilm formation, Antibiotic resistance

Introduction

For up to 6000 years, Egyptians have ranched goats and sheep and consumed their milk.[1] The use of goat and sheep milk for medicinal purposes and household consumption is prominent throughout the world. Herd survivability, management ease, and affordability promote the ranching of goats primarily in low to middle-income countries.[2] In locations with little availability to cattle or other alternate milk, populations with prominent milk allergies, or increased exposure to milk-borne pathogens such as *Listeria*, it is necessary to consider alternative milk. Goat and ewe milk has been proposed as an alternative, nutrient-rich milk to feed infants allergic to cow's milk.[3]

Listeria species are ubiquitous; however, only two of 10 species, *Listeria monocytogenes* and *Listeria ivanovii*, are pathogenic in human populations.[4] *Listeria monocytogenes* is a foodborne pathogen that may cause a severe, invasive illness with a corresponding mortality rate up to 30%;[5] furthermore, exposure to *L. monocytogenes* is second only to salmonellosis among the most frequent causes of death due to a foodborne illness.[6]

Among animals, listeriosis may affect sheep and goats and the pathology may develop in different clinical forms and include mastitis.[7] The excretion of *Listeria* in milk may persist throughout lactation and contribute to an increased risk of milk product contamination.[6] Sheep milk has previously been

Corresponding author: Tara Zolnikov, North Dakota State University, Developmental Science, Fargo, ND 58102, USA. Email: tarazolnikov@gmail.com

Table 1   Cultural and biochemical differentiation between the *Listeria* species recovered from udder raw fresh whole milk of goat and sheep

Phenotypic characteristics	*Listeria monocytogenes*; reference strain (ATCC 7494)	*L. monocytogenes*	*L. seeligeri*	*L. innocua*	*L. welshimeri*	*L. grayi*
			Listeria species			
ALOA agar	Blue green with halo	Blue green with halo	Blue green without halo	Blue green without halo	Blue green without halo	Blue green without halo
Hemolysis	beta-hemolysis	beta-hemolysis	beta-hemolysis	Non-hemolytic	Non-hemolytic	Non-hemolytic
Motility	Motile at 22–25°C	Motile at 22–25°C	Motile at 37°C	Motile at 37°C	Motile at 37°C	Motile at 37°C
Nitrate reduction test	–	–	–	–	–	+
D-Xylose fermentation	–	–	+	–	+	–
L-Rhamnose fermentation	+	+	–	Variable	Variable	Variable
D-Mannitol	–	–	–	–	–	+
Alpha methyl D-mannoside	+	+	–	+	+	+
Genus *Listeria* distinguished on the basis of the 16S rRNA	+	+	+	+	+	+
Rapid detection of *L. monocytogenes* in milk using *hly*A gene	+	+	+	–	–	–

described as a possible cause for listeriosis.[8] Intermittent shedding of *L. monocytogenes* has been reported in goats and sheep without clinical signs of listeriosis.[9,10] Therefore, the identification of infected animals was necessary due to the likely causal link to several outbreaks of listeriosis.[8–12]

Currently, studies confirming the virulence potential and polymorphism of *L. monocytogenes* strains prevalent in Egypt neither exist nor have been previously conducted. This study sought to compare milk related *Listeria* isolates of two domesticated animals, goats and sheep. Prevalence, biochemical and serological characterization, *in vitro* and *in vivo* pathogenicity, and antimicrobial resistance profiles were assessed. The *plc*B, *act*A, *iap*, and *hly*A genes for *L. monocytogenes* were aptly chosen as target genes throughout this study.[13]

Materials and Methods

The study took place in private dairy farms surrounding Cairo, Egypt where 209 milk samples from the mammary glands of sheep (n = 102) and goats (n = 107) were collected. The animals had not been treated with an antibiotic for at least 30 days prior to collection. Milk samples were screened for mastitis using the California mastitis test (CMT) prior to the isolation of *Listeria* spp. Milk samples were taken under aseptic conditions for bacteriological examinations. A subsample of 15 ml of milk, taken from a composite milk sample, was collected in sterile universal bottles. The milk samples were quickly transported to the laboratory under chilled conditions and stored at 4°C till bacteriologically analyzed.

For the isolation and identification of *Listeria* species in the milk samples, the techniques recommended by the International Organization for Standards (ISO 11290-1, 1996) were implemented.

Isolation and identification of *Listeria* was performed using the double enrichment procedure, the first (Half Fraser) and the second (Fraser) enrichment broths. A loopful of growth from Fraser II was subcultured onto Palcam selective agar supplemented with SR 0150E (polymyxin B 5 mg, acriflavine HCl 2.5 mg) (Oxoid) and incubated micro-aerobically (5% O_2, 10% CO_2, and 85% N_2). Colonies suspected to be *Listeria* were transferred onto tryptic soya yeast extract agar (TSYEA) (Difco, Bacton, PA, USA) and incubated at 30°C for 18–24 hours. Those putative *Listeria* colonies were tested for purity, in addition to morphological and biochemical characteristics (Table 1). Strains were also identified using the API® *Listeria* system (bioMe'rieux, Marcy l'Etoile, France) and the Oxoid Microbact™ *Listeria* 12L (MB1128A). Presumptive *Listeria* colonies were maintained at 4°C on Trypticase soy agar with 0.6% yeast extract slants, incubated at 37°C for 24 hours, and stored at 4°C for final serotyping and PCR confirmation.

Serotyping was carried out on *L. monocytogenes* strains using commercial specific antisera (Behringwerke AG, Marburg, Germany) against the serovars 1and 4, following the manufacturer's instructions. All purified isolates were tested by the standard disc diffusion method (CLSI, 2010) and were subjected to a susceptibility panel of 28 antibiotics (Oxoid) belonging to 11 drug classes (Table 6). 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°C for 48 hours

Table 2 Pathogenicity profiles of *Listeria monocytogenes* recovered from udder raw fresh whole milk of goat and sheep

Source of milk samples	Serotype	Pathogenicity profile								Biofilm		PCR profile of virulence associated genes			
		CAMP (±)	PI-PLC	DLABN	DAPN	Anton's eye test	Mice lethality	Chick-embryo lethality	Vero cell ingestion assay	Christensen's tube	Microtiter plate assay	*hlyA*	*plcB*	*actA*	*iap*
Reference strain (ATCC 7494)	4	±	+	−	−	+	+	+	++	Strong	Strong (OD = 0.158)	+	+	+	+
Goat	1	+/+	+	−	−	+	+	+	++	Moderate	Very strong (OD = 0.201)	+	+	+	+
	4	±	+	−	−	+	+	+	++	Strong	Strong (OD = 0.104)	+	+	+	+
Ewe	4	±	+	−	−	+	+	+	++	Strong	Strong (OD = 0.117)	+	+	+	+

CAMP: Christie, Atkins, Munch-Petersen test; S/R: *Staphylococcus aureus*/*Rhodococcus equi*; PI-PLC: phosphatidylinositol-specific phospholipase C; DLABN: DL-alanine- beta-naphthylamide HCl; DAPN: D-alanine-*p*-nitroanilide; OD: optical density; $OD_{595} < 0.1$ = weak; $OD_{595} \leq 0.1$ = strong; $OD_{595} > 1$ = very strong.

Table 3 Pathogenicity profiles of *Listeria* species recovered from udder raw fresh whole milk of goat and sheep

Source of milk samples	*Listeria* species	Pathogenicity profile								Biofilm		PCR profile of virulence associated genes			
		CAMP (±) with S/R	PI-PLC	DLABN	DAPN	Anton's eye test	Mice lethality	Chick-embryo lethality	Vero cell ingestion assay	Christensen's tube	Microtiter plate assay	*hlyA*	plc*B*	*actA*	*iap*
	L. monocytogenes (ATCC 7494)	±	+	−	−	+	+	+	+	Strong	Strong (OD = 0.158)	+	+	+	+
Goat	*L. innocua*	−	−	+	+	−	−	−	−	Weak	Strong (OD = 0.159)	−	−	−	−
	L. seeligeri	−	−	+	+	−	−	−	−	Moderate	Strong (OD = 0.221)	+	−	−	−
	L. welshimeri	−	−	+	+	−	−	−	−	Moderate	Very strong OD = 0.112)	−	−	−	−
Sheep	*L. innocua*	−	−	+	+	−	−	−	−	Moderate	Strong (OD = 0.107)	−	−	−	−
	L. grayi	−	−	+	+	−	−	−	−	Weak	Strong (OD = 0.127)	−	−	−	−
	L. grayi	−	−	+	+	−	−	−	−	Moderate	Strong (OD = 0.126)	−	−	−	−

CAMP: Christie, Atkins, Munch-Petersen test; S/R: *Staphylococcus aureus*/*Rhodococcus equi*; PI-PLC: phosphatidylinositol-specific phospholipase C; DLABN: DL-alanine-beta-naphthylamide HCl; DAPN: D-alanine-*p*-nitroanilide; OD: optical density; OD < 0.1 = weak; OD 0.1 = strong; OD > 0.1 = very strong.

Table 4 Prevalence of *Listeria* species recovered from udder raw fresh whole milk of goat and sheep

Animal species	Health condition of the animals	Number of examined samples	*L. monocytogenes* n	%	*L. innocua* n	%	*L. welshimeri* n	%	*L. seeligeri* n	%	*L. grayi* n	%	Total n	%
Goat	Apparent Healthy animal	33	0	0	0	0	1	3.0	0	0	0	0	1	3.0
	Subclinical Mastitic animal	35	1	2.9	1	2.9	0	0	1	2.9	0	0	3	8.6
	Clinical Mastitic animal	39	1	2.6	0	0	0	0	1	2.6	0	0	2	5.1
	Total	107	2	1.9	1	0.9	1	0.9	2	1.9	0	0	6	5.6
Sheep	Apparent Healthy animal	38	0	0	0	0	0	0	0	0	1	2.6	1	2.6
	Subclinical Mastitic animal	41	1	2.4	0	0	0	0	0	0	1	2.4	2	4.9
	Clinical Mastitic animal	23	0	0	1	4.4	0	0	0	0	0	0	1	4.4
	Total	102	1	1.0	1	1.0	0	0	0	0	2	2.0	4	3.9
Total		209	3	1.4	2	1.0	1	0.5	2	1.0	2	1.0	10	4.8

All the biochemically characterized *Listeria* isolates were assayed for phosphatidylinositol-specific phospholipase C (PI-PLC) activity,[14] and DL-alanine-beta-naphthylamide HCl (DLABN) and D-alanine-*p*-nitroanilide (DAPN) tests further differentiated between *L. monocytogenes* and other species of *Listeria* Clark and McLauchlin (1997).[15] The classical tests for Listeria pathogenicity implemented were the Anton conjunctivitis test (rabbits), inoculation of mice, inoculation of embryonated eggs, and ingestion assay on Vero cells.[16,17] A qualitative assessment of the biofilm formation assay was developed according to Giacaman *et al.* (2010) through the Christensen's tube method and by the tissue culture plate assay, as described by Borucki *et al.* (2003).[18,19]

The PCR was used for the detection of the genus *Listeria*. Freshly grown typical *Listeria*-like colonies (black colonies) from the surfaces of Palcam plates were boiled in 400 μl of 1× Tris-EDTA buffer (pH 8.0) for 10 minutes and centrifuged at 16 873 g for 10 minutes to remove denatured proteins and bacterial membranes. *Listeria* was distinguished on the basis of the 16S rRNA that can be revealed by PCR (U1forward primer 5-CAG-CMG-CCG-CGG-TAA-TWC-3, where M denotes A or C and W denotes A or T and LI1 reverse primer 5-CTC-CAT-AAA-GGT-GAC-CCT-3) to target a 938-bp 16S rRNA sequence in members of the genus *Listeria*.[20,21] Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The samples were subjected to an initial denaturation step of 94°C for 4 minutes, followed by 25 amplification cycles of 1 minute at 94°C (denaturation), 1 minute at 60°C (primer annealing), and 1 minute at 72°C (primer extension) followed by a final extension step of 72°C for 5 minutes. PCR reaction products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized. An *L. monocytogenes* strain (ATCC 7494) and an *E. coli* strain (ATCC 25922) were included as positive and negative controls respectively.

For PCR detection of virulence genes among *Listeria* species, the *Listeria* pellet collected from the surfaces of Palcam plates was dispersed in 400 μl 10 mM Tris-1 mM EDTA buffer containing 0.1% Tween 80 and 150 μl (2 mg/ml) lysozyme and incubated for 15 minutes on ice. The pellet was lysed by redissolving in TE buffer containing 40 μl (100 μg of Proteinase K/ml) Proteinase K and 60 μl (10% (w/v)) sodium dodecyl sulfate and incubated for 30 minutes at 37°C. DNA was extracted by adding an equal volume of TE saturated phenol: chloroform: iso-amyl alcohol (25 : 24 : 1 v/v/v) and precipitated by the addition of 20 μg of acrylamide/ml, 0.05 volumes of 3M sodium acetate and 2.5 volumes of ethanol.

Table 5 Serotyping of *Listeria monocytogenes* recovered from udder raw fresh whole milk of goat and sheep

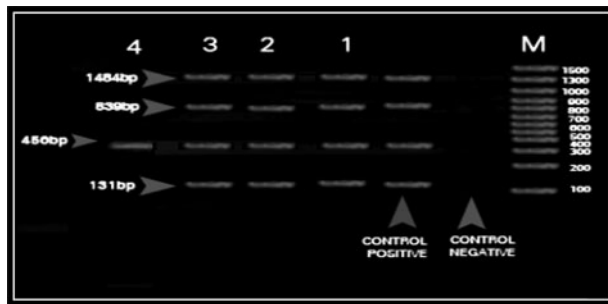| Animal species	Number of *L. monocytogenes* isolates	Type 1 n	%	Type 4 n	%	Untyped n	%
Goat milk	2	1	50	1	50	0	0
Sheep milk	1	0	0	1	100	0	0
Total	3	1	33.3	2	66.7	0	0

Figure 1 **Multiplex PCR of four virulence associated genes for standard *Listeria monocytogenes* (ATCC 7494) and isolated *Listeria* species. M: DNA marker (100–1500 bp); control negative: *E. coli* strain ATCC 25922; control positive: amplified products of four virulence associated genes of standard *L. monocytogenes* (ATCC 7494) – *plc*B gene (1484 bp); *act*A gene (839 bp); *hly*A gene (456 bp); *iap* gene (131 bp); Lane 1: amplified products of four virulence associated genes of the isolated *L. monocytogenes*; Lane 2: amplified products of one virulence associated gene *hly*A gene (456 bp) of the isolated *Listeria seeligeri*.**

The 10 goat and ewe *Listeria* isolates were screened by PCR for the presence of the virulence genes using the primers: *hly*A (for.) 5′-GCA GTT GCA AGC GCT TGG AGT GAA-3′, (rev.) 5′- GCA ACG TAT CCT CCA GAG TGA TCG-3′ to amplify the 456-bp fragment;[22] *plc*B (for.) 5′-CTG CTT GAG CGT TCA TGTCTC ATC CCC C-3′, (rev.) 5′-ATG GGT TTC ACT CTCCTT CTA C-3′ to amplify the 1484-bp fragment;[23] *act*A (for.) 5′-CGC CGC GGA AATTAA AAA AAG A-3′, (rev.) 5′- ACG AAG GAA CCG GGCTGC TAG - 3′ to amplify the 839-bp fragment; and *Iap* (for.) 5′-ACA AGC TGC ACC TGT TGC AG-3′, (rev.) 5′-TGACAG CGT GTG TAG TAG CA-3′ to amplify the 131-bp fragment.[24] Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The samples were subjected to an initial denaturation step of 95°C for 2 minutes, followed by 35 amplification cycles of 15 seconds at 95°C (denaturation), 30 seconds

Table 6 **Result of antimicrobial susceptibility for *Listeria* species isolated from fresh udder milk**

Antibiotics	Species of isolated *Listeria*		
	Listeria monocytogenes reference strain (ATCC7494)	*L. monocytogenes*	Other *Listeria* species
Penicillins			
Ampicillin (25 μg)	1/1	3/3	1/7
Penicillin G (10IU)	1/1	3/3	3/7
Amoxicillin/clavulanic acid (10 μg)	1/1	3/3	1/7
Cloxacillin (5 μg)	0/1	0/3	0/7
Oxacillin (1 μg)	0/1	0/3	0/7
Amoxicillin (25 μg)	1/1	3/3	1/7
Fluoroquinolones			
Ofloxacin (10 μg)	1/1	3/3	7/7
Enrofloxacin (10 μg)	1/1	3/3	7/7
Ciprofloxacin (5 μg)	1/1	3/3	1/7
Flumequine (30 μg)	0/1	0/3	0/7
Pefloxacin (30 μg)	0/1	0/3	0/
Aminoglycosides			
Amikacin (30 μg)	1/1	3/3	7/7
Gentamicin (10 μg)	1/1	3/3	7/7
Kanamycin (30 μg)	1/1	3/3	7/7
Neomycin (10 μg)	1/1	3/3	7/7
Streptomycin (10 μg)	1/1	3/3	1/7
Phenicol			
Chloramphenicol (30 μg)	1/1	2/3	7/7
Tetracycline			
Tetracycline (30 μg)	1/1	2/3	7/7
Sulfonamide			
Trimethoprim-sulfamethoxazole 1 : 19 (25 μg)	1/1	2/3	1/7
Cephalosporins			
Cefotaxime (30 μg)	0/1	0/3	0/7
Cephalothin (30 μg)	0/1	0/3	0/7
Lincosamides			
Lincomycin (2 μg)	0/1	0/3	0/7
Clindamycin (2 μg)	0/1	0/3	0/7
Polypeptides			
Bacitracin (10 units)	0/1	0/3	0/7
Glycopeptide			
Vancomycin (30 μg)	1/1	3/3	7/7
Macrolide			
Erythromycin (15 μg)	1/1	3/3	7/7
Spiramycin (100 μg)	1/1	3/3	7/7
Rifamycin			
Rifampicin (5 μg)	1/1	3/3	2/7

at 60°C (annealing), and 90 seconds at 72°C (primer extension), followed by a final extension step of 72°C for 10 minutes. Visualization of the PCR products was carried out as previously indicated (Fig. 1).

The study was performed at the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt in accordance with the recommendations in the updated Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NRC, 2010).

Results

A total of 209 milk samples collected from goats (107) and sheep (102) were screened for *Listeria* species (Table 4). The microbiological analysis revealed an overall prevalence of 10 (goat, 6/107; sheep, 4/102) putative *Listeria* spp isolates (10/209; 4.8%). Out of these, three isolates (goats, 2/6; sheep 1/4) were identified as *L. monocytogenes* (3/10; 30.0%). The remaining seven were considered *Listeria* species. In the 107 goat udder milk samples, two were positive for *L. monocytogenes* (1.9%); while in the 102 ewe udder milk samples, only one sample was positive for *L. monocytogenes* (1.0%). The overall prevalence of Listeria species were found to be: *Listeria* innocua (2/209; 1.0%), *L. welshimeri* (1/209; 0.5%), L. seeligari (2/209; 1,0%), and *Listeria grayi* (2/209; 1.0%). The three *L. monocytogenes* isolated from 209 milk samples (3/209; 1.4%) were serologically typed as either identified *L. monocytogenes* serotype Type 1 (1/1) isolated from the goat sample or Type 4 isolated from samples obtained from both goat (1/2) and sheep (1/1) milk samples (Table 5). The *L. monocytogenes* isolate from all three samples (goat, 2/6; sheep, 1/4) possessed all the four virulence associated genes while *L. seeligeri* carried the *hly*A gene only.

The milk samples did not appear visibly abnormal throughout the course of the study. In total, 107 goat milk samples were collected; 33 were normal, 35 were subclinical mastitic, and 33 were clinically mastitic. The 102 milk samples collected from sheep were comprised of 23 clinically mastitic, 41 subclinically mastitic, and 38 normal milk samples.

All tested isolates were small, Gram-positive rods, negative for indole, oxidase, urease, and H$_2$S production from organic sulfur compounds and test-positive for methyl red and Voges–Proskauer, had the ability to grow at 35°C, were catalase-positive and motile in wet mounts indicative for all Listeria species. They utilized dextrose, esculin, maltose, and some species utilized rhamnose and xylose with production of acid. The only isolate that utilized mannitol with acid production was L. grayi, while L. monocytogenes and L. seeligeri produced hemolysis in sheep blood stabs and consequently were CAMP test-positive (Table 1). Of the two, only

L. monocytogenes failed to utilize xylose and was positive for rhamnose utilization. The researchers observed that the non-hemolytic species L. innocua and L. grayi provided the same xylose reactions as L. monocytogenes but were negative in the CAMP test. *Listeria* innocua gave a variable result for the utilization of rhamnose, an observation that was also recorded in the present investigation with L. welshimeri and L. grayi (Table 1).

The pathogenicity testing of the 10 *Listeria* isolates via the PI-PLC, DLABN, and DAPN assays, as well as *in vivo* tests, namely chick embryo, mice inoculation tests, Anton's eye test, and Vero ingestion assay, indicated that three hemolytic *L. monocytogenes* isolates were determined to be pathogenic (Table 2). Splenic samples from deceased mice of the *L. monocytogenes* challenge contained viable *L. monocytogenes* that were recovered on BHI agar and confirmed by PCR; conversely, splenic samples from surviving mice had no viable *L. monocytogenes* detectable on BHI agar. The three hemolytic isolates of *L. monocytogenes* showed the characteristic enhancement of hemolytic zone with *Staphylococcus aureus* (Tables 1 and 2). All other *Listeria* spp. isolates were non-pathogenic (Table 3).

The results of adherence assay to test glass tube assessed by 0.1% Crystal Violet stain showed that the three (1/2 goat and 1/1 sheep) *L. monocytogenes* isolates were able to strongly form a biofilm on a glass surface, while one of the two isolates from the goat was moderately adherent (Table 2). L. innocua, *L. welshimeri*, L. seeligari, and *L. grayi* varied in their adherence and biofilm formation (Table 3). The three *L. monocytogenes* strains were screened for their adherence to polystyrene 96-well microtiter plates at different degrees; the results conclude *L. monocytogenes* was able to form a strong biofilm on polystyrene (OD$_{570}$ > 1) (Table 2). The same strong affinity was also observed with the seven (goats, 4/6; sheep, 3/4) *Listeria* species, L. innocua (goat 1/6; sheep, 1/4), *L. welshimeri* (goat, 1/6), L. seeligari (goat, 2/6), and *L. grayi* (sheep, 2/4) (Table 3).

Discussion

From the results, Listeria was distinguished from Brochothrix, which is of concern as a food spoilage organism, by its inability to grow at 35°C and its lack of motility.[17] From the five species isolated, the only isolate that utilized mannitol with acid production was L. grayi.[17] It was also confirmed that L. monocytogenes and L. seeligeri produced hemolysis in sheep blood stabs, as also determined by Hitchins and Jinneman (2013).[17] Prior research has determined that L. innocua may produce negative results for the utilization of rhamnose;[17] this observation was also recorded in the present investigation with L. welshimeri

and L. grayi (Table 1). After confirming negative results for the utilization of rhamnose, serotyping of Listeria isolates was differentiated. Listeriolysin O (LLO) is the main virulence factor and exists in all of the pathogenic *L. monocytogenes* coded by *hly*A gene; according to a study by Choi and Hong,[51] the *hly*A gene of *L. monocytogenes* would be able to determine two cells in milk through a PCR assay (Tables 1–3). Although *L. ivanovii* is predominantly an animal pathogen affecting ruminants and sheep,[20,25] it was not isolated in this study.

Listeria monocytogenes isolated from sheep milk in animals suffering from subclinical mastitis was characterized by a persistent shedding of *Listeria* and an apparently normal milk consistency and appearance.[26–29] *Listeria* spp. was never found in sheep subclinical mastitis without obvious inflammation. L. seeligeri was isolated from 1.67% of healthy-appearing goat fecal samples.[29–31]

Animals may become infected through the ingestion of *Listeria*-contaminated food, poor-quality silage, pathogen inhalation, direct contact to the source, drinking water, feed components, soil in which fodder plants were grown, silage feeding, sawdust bedding, and farm yard manure;[32–38,39] therefore, distributing untreated manure for agricultural purposes is regarded as a primary risk factor for the transmission of *Listeria*, thereby contributing to the spread of foodborne disease.[37]

Literature confirming the virulence of associated *Listeria* genes isolated from sheep and goats currently does not exist. With the exception of *L. seeligeri* being hemolytic, though non-pathogenic, the only pathogenic strain of *L. monocytogenes* is hemolytic. Variations in the degree of hemolysis among the bacteria have also been reported by Rawool *et al.* (2007).[12] It has also been demonstrated that the *L. monocytogenes* phospholipases are essential determinants of pathogenicity.[38,39] These studies provided evidence for the virulence factor activity of PI-PLC, expressed only by pathogenic spp. of *Listeria* i.e., *L. monocytogenes*.[40,41] This expression remains a reliable marker for the discrimination between pathogenic and non-pathogenic *Listeria* species.[40,41] The positivity of the *L. monocytogenes* in PI-PLC assay may be explained by the common regulation of the *hly*A, *act*A, and *plc*B genes.[42]

Genetic tests that have been used for the detection and/or identification of *Listeria* species have exploited the molecular differences of the 16S and 23S rRNA genes.[43] Polymerase chain reaction assays have also been developed to target specific virulence genes such as the *hly*A gene (part of LIPI-1), which are present in specific *Listeria* species alongside the virulence gene, *iap,* that is common to all *Listeria* species.[44,45] *Listeria monocytogenes* is pathogenic at

the species level, but various strains display varied virulence and pathogenic potential. Some strains may be virulent and cause disease while other strains may remain non-pathogenic and produce no apparent malaise.[44,46,47,48] The difference between these virulent and non-virulent strains is likely minimal, as they possess the same virulence gene cluster LIPI-1 (*prf*A, *hly*A, *plc*A, *mp*I, *act*A, and *plc*B) and major virulence proteins involved in *L. monocytogenes* pathogenesis, but may differ in the expression of unique genes.[44] LIPI-1 includes the genes *prf*A (encodes positive regulatory factor A), *plc*A (PI-PLC), *hly*A (hemolysin LLO), *mpl* (zinc metalloprotease), *act*A (surface actin polymerization protein), *plc*B (phosphatidylcholine phospholipase C (PC-PLC)), and the *iap* (invasion associated protein) encodes the protein p60. The latter gene can be used to distinguish between the different *Listeria* species as it is only present in *L. ivanovii, L. seeligeri*, and *L. monocytogenes*.[49] The gene *plcA,* which encodes PI-PLC, however, is only present in *L. ivanovii* and *L. monocytogenes*. Phosphatidylinositol-specific phospholipase C and LLO acts in synergy to destroy the phagosome in which the bacterium is trapped during infection. This is one of four major steps involved in the cellular mechanism of *Listeria* pathogenesis. The absence of the *plcA* gene in *L. seeligeri* is, therefore, a contributing factor to its non-pathogenic status.[49] The *hly*A gene, is responsible for the hemolysis of blood cells[48] and is only found in virulent strains of *L. monocytogenes*. The PCR employed in the present study was specific for individual detection of the four virulence associated genes *plc*B, *act*A, *hly*A, and *iap* found in *L. monocytogenes* while *L. seeligeri* had an intact *hly*A gene only. These findings commensurate with the former scientific research for the detection of *hly*A gene,[22] *plc*B gene,[14] *iap* gene, and *act*A gene.[24] Although Gouin *et al.* (1994) and Bhunia (2008) assumed that the *plc*B could be used to distinguish between the different *Listeria* species in *L. ivanovii, L. seeligeri*, and *L. monocytogenes*, it was not detected in the two isolated strains of *L. seeligeri* in the present investigation.[50] As LLO is a secreted protein, the detection of it in a food sample can also be an indicator of the presence of *L. monocytogenes* cells.[20]

Conclusion

This study concludes a low, though viable, prevalence of *L. monocytogenes* in both sheep and goat milk. Human exposure can occur through the consumption of raw, unpasteurized milk. Pregnant women should undergo health and safety precautions in milking goats and sheep, especially those animals that have recently given birth; pregnant women should also avoid coming into contact with the afterbirth or with newborn lambs. Immunosuppressed individuals are

advised to undertake similar precautions. It is important for human populations to maintain strict hygiene and practice pasteurization of raw milk in order to minimize the risks of human infection with *L. monocytogenes*.

Conflict of Interest

The authors declare no personal or financial conflicts of interests in the development, writing, and submission of this study.

References

1 Herodotus 440 B.C. The History of Herodotus By Herodotus Written 440 B.C.E Translated by George Rawlinson Book II Provided by The Internet Classics Archive. Available http://classics.mit.edu//Herodotus/history.html
2 Haenlein GFW. Goat milk in human nutrition. Small Ruminant Res. 2004;51:155–63.
3 Camejo A, Carvalho F, Reis O, Leitão E, Sousa S, Cabanes D. The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. Virulence. 2011;2:379–94.
4 Rebagliati V, Philippi R, Rossi M, Troncoso A. Prevention of foodborne listeriosis. Indian J Pathol Microbiol. 2009;52:145–9.
5 Kasalica A, Vuković V, Vranješ A, Memiši N. *Listeria monocytogenes* in milk and dairy products. Biotechnol Anim Husb. 2011;27:1067–82.
6 Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev. 1991;55:476–511.
7 Fthenakis GC, Saratsis Ph, Tzora A, Linde K. Naturally occurring subclinical ovine mastitis associated with *Listeria monocytogenes*. Small Ruminant Res. 1998;31:23–7.
8 Gronstol H. Listeriosis in sheep. *Listeria monocytogenes* excretion and immunological state in healthy sheep. Acta Vet Scand. 1979;20:168–79.
9 Sharp MW. Bovine mastitis and *Listeria monocytogenes*. Vet Rec. 1989;125:512–3.
10 Conly JM, Johnston BL. *Listeria*: a persistent food-borne pathogen. Can J Infect Dis Med Microbiol. 2008;9:327–8.
11 MMWR. Outbreak of *Listeria monocytogenes* infections associated with pasteurized milk from a local dairy – Massachusetts, 2007. MMWR Morb Mortal Wkly Rep. 2008;57:1097–100.
12 Rawool DB, Malik SVS, Shakuntala I, Sahare AM, Barbuddhe SB. Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases. Int J Food Microbiol. 2007;113:201–7.
13 Notermans SHW, Dufrenne J, Leimeister-Wachter M, Domann E, Chakraborty T. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. Appl. Environ. Microbiol. 1991;57:2666–70.
14 Clark AG, McLauchlin J. Simple color tests based on an alanyl peptidase reaction which differentiate *Listeria monocytogenes* from other *Listeria* species. J Clin Microbiol. 1997;35:2155–6.
15 Raja RK, Ramesh N, Maripandi A. Invasion and interaction studies of *Salmonella* Typhimurium sub sp enteritis in vero and MDCK cell lines. Adv Biol Res. 2010;4:86–91.
16 Hitchins AS, Jinneman K. 2013. Bacteriological analytical manual, chapter 10 detection and enumeration of *Listeria monocytogenes* in foods. Silver Spring, MD, USA: U.S. Food and Drug Administration, Page Last Updated: 2013 February 14.
17 Giacaman RA, Araneda E, Padilla C. Association between biofilm forming isolates of mutans *Streptococci* and caries experience in adults. Arch Oral Biol. 2010;55:550–4.
18 Borucki MK, Peppin JD, White D, Loge F, Call DR. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol. 2003;69:7336–42.
19 Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, *et al*. *Listeria* pathogenesis

and molecular virulence determinants. Clin Microbiol Rev. 2001;14:584–640.
20 Wesley IV, Harmon KM, Dickson JS, Schwartz AR. Application of a multiplex polymerase chain reaction assay for the simultaneous confirmation of *Listeria monocytogenes* and other *Listeria* species in turkey sample surveillance. J Food Prot. 2002;65:780–5.
21 Paziak-Domanska B, Bogulawska E, Wiekowska-Szakiel M, Kotlowski R, Rozalska B, Chmiela M, *et al*. Evaluation of the API test, phosphatidylinositol-specific phospholipase C activity and PCR method in identification of *Listeria monocytogenes* in meat foods. FEMS Microbiol Lett. 1999;171:209–14.
22 Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MU, Dharsana KS, Prasad SP, *et al*. Listeria: review of epidemiology and pathogenesis. J Microbiol Immunol Infect. 2007;40:4–13.
23 Barza M. Listeriosis and milk. N Engl J Med. 1985;312:438–40.
24 Schoder D, Winter P, Kareem A, Baumgartner W, Wagner M. A case of sporadic ovine mastitis caused by *Listeria monocytogenes* and its effect on contamination of raw milk and raw-milk cheeses produced in the on-farm dairy. J Dairy Res. 2003;70:395–401.
25 Winter P, Schilcher F, Bagò Z, Schoder D, Egerbacher M, Baumgartner W, *et al*. Clinical and histopathological aspects of naturally occurring mastitis caused by *Listeria monocytogenes* in cattle and ewes. J Vet Med B Infect Dis Vet Public Health. 2004;51:176–9.
26 Pintado CM, Grant KA, Halford-Maw R, Hampton MD, Ferreira MA, McLauchlin J. Association between a case study of asymptomatic ovine listerial mastitis and the contamination of soft cheese and cheese processing environment with *Listeria monocytogenes* in Portugal. Foodborne Pathog Dis. 2009;6:569–75.
27 Elezebeth G, Malik SVS, Chaudhari SP, Barbuddhe SB. The occurrence of *Listeria* species and antibodies against listeriolysin-O in naturally infected goats. Small Ruminant Res. 2007;67:173–8.
28 Fotou K, Tzora A, Voidarou Ch, Alexopoulos A, Plessas S, Avgeris I, *et al*. Isolation of microbial pathogens of subclinical mastitis from raw sheep's milk of Epirus (Greece) and their role in its hygiene. Anaerobe. 2011;17:315–9.
29 De Luca G, Zanetti F, Fateh-Moghadm P, Stampi S. Occurrence of *Listeria monocytogenes* in sewage sludge. Zentralbl Hyg Umweltmed. 1998;201:269–77.
30 Yoshida T, Kato Y, Sato M, Hirai K. Sources and routes of contamination of raw milk with *Listeria* monocytogenes and its control. J Vet Med Sci. 1998;60:1165–8.
31 Ryser ET, Marth EH (editors). *Listeria*, listeriosis, and food safety. CRC Press; 2007.
32 Zundel E, Bernard S. *Listeria monocytogenes* translocates throughout the digestive tract in asymptomatic sheep. J Med Microbiol. 2006;55:1717–23.
33 Brugère-Picoux J. Ovine listeriosis. Small Ruminant Res. 2008;76:12–20.
34 Hellstrom S. Contamination routes and control of *Listeria monocytogenes* in food production. Finland: Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki; 2011. [Helsinki University Print].
35 Latorre AA, Van Kessel JAS, Karns JS, Zurakowski MJ, Pradhan AK, Boor KJ, *et al*. Increased *In vitro* adherence and on-farm persistence of predominant and persistent *Listeria monocytogenes* strains in the milking system. Appl Environ Microbiol. 2011;77:3676–84.
36 Behravesh CB, Williams IT, Tauxe RV. Emerging foodborne pathogens and problems: expanding prevention efforts before slaughter or harvest. In: Institute of Medicine (US). Improving food safety through a one health approach: workshop summary. Washington, DC: National Academies Press; 2012. p. A14. Available from: http://www.ncbi.nlm.nih.gov/books/NBK114501/
37 Oliver SP, Jayarao BM, Almeida RA. Foodborne pathogens, mastitis, milk quality, and dairy food safety. NMC Annual Meeting Proceedings, Orlando, Florida, USA; 2005.
38 Marquis H, Doshi V, Portnoy DA. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. Infect Immun. 1995;63:4531–4.
39 Smith GA, Marquis H, Jones S, Johnston NC, Portnoy DA, Goldfine H. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect Immun. 1995;63:4231–7.

40 Liu D. *Listeria monocytogenes*: comparative interpretation of mouse virulence assay. FEMS Microbiol Lett. 2004;233:159–64.

41 Graham T, Golsteyn-Thomas EJ, Gannon VP, Thomas JE. Genus- and species-specific detection of *Listeria monocytogenes* using polymerase chain reaction assays targeting the 16S/23S intergenic spacer region of the rRNA operon. Can J Microbiol. 1996;42:1155–62.

42 Graham TA, Golsteyn-Thomas EJ, Thomas JE, Gannon VP. Inter- and intraspecies comparison of the 16S–23S rRNA operon intergenic spacer regions of six *Listeria* spp. Int J Syst Bacteriol. 1997;47:863–9.

43 Sallen BA, Rajoharison S, Desverenne S, Quinn F, Mabilat C. Comparative analysis of 16S and 23S rRNA sequences of *Listeria* species. Int J Syst Bacteriol. 1996;46:669–74.

44 Blais BW, Turner G, Sooknanan R, Malek LT. A nucleic acid sequence based amplification system for detection of *Listeria monocytogenes hly*A sequences. Appl Environ Microbiol. 1997;63:310–3.

45 Bubert A, Hein I, Raunch M, Lehner A, Yoon B, Goebel W, *et al*. Detection and differentiation of *Listeria* spp. by a single reaction based on multiplex PCR. Appl Environ Microbiol. 1999;65:4688–92.

46 Erdenlig S, Ainsworth AJ, Austin FW. Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. J Food Prot. 2000;63:613–9.

47 Gracieux P, Roche SM, Pardon P, Velge P. Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid' L. mono media. Int J Food Microbiol. 2003;83:133–45.

48 Doumith M, Cazalet C, Simoes N, Frangeul L, Jacquet C, Kunst F, *et al*. New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. Infect Immun. 2004;72:1072–83.

49 Kingdon GC, Sword P. Effect of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes. Infect Immun. 1970;1:356–62.

50 Bhunia AK. *Listeria monocytogenes*. In: Foodborne microbial pathogens. New York: Springer Science + Business Media; 2008. p. 165–82.

51 Choi WS, Hong C-H. Rapid enumeration of *Listeria mono-cytogenes* in milk using competitive PCR. Int J Food Microbiol. 2003;84:79–85.