

## Research Article

# Characterization and Antimicrobial Susceptibility Patterns of *Listeria monocytogenes* from Raw Cow Milk in the Southern Part of Ethiopia

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Food safety remains the main health concern in the developing countries. Thus, the major purpose of the present study was to characterize and determine antibiotic susceptibility patterns of *Listeria monocytogenes* from raw milk samples collected from southern Ethiopia. Two hundred and forty raw cow milk samples were collected from dairy farms and smallholder dairy producers using a simple random sampling technique and analyzed by cultural and multiplex PCR methods. The antimicrobial susceptibility profile of *L. monocytogenes* was evaluated using the standard disk diffusion method. Over 28% of the samples were found positive for *Listeria* spp., of which 17 (7.08%) isolates were identified as *L. monocytogenes* after morphological and biochemical confirmation. The prevalence of *L. monocytogenes* was 6.02% in Hawassa city, 5.56% in Dale district, and 9.41% in Arsi Negele district. *L. monocytogenes* was higher in the wet season (9.32%) than in the dry season (4.92%). The gene for *Listeria* specific 16S rRNA was detected in all the 17 examined isolates, while *hlyA* and *iapA* were only found in 11 of them. Furthermore, no isolate was identified to have the *prfA*, *actA*, or *plcA* genes. Antimicrobial resistance profiling revealed that all the *L. monocytogenes* isolates were resistant to nalidixic acid (100%), followed by erythromycin (88.24%). However, all the *L. monocytogenes* isolates were sensitive to vancomycin, gentamicin, and sulfamethoxazole. Raw cow milk is a potential source of *L. monocytogenes* and it poses a threat to human and animal health. Therefore, it is crucial that dairy producers and vendors of raw milk in the study areas should take considerable precautions to prevent *Listeria* species from contaminating raw fresh milk.

## 1. Introduction

Listeriosis is a significant bacterial zoonotic disease caused by *Listeria monocytogenes*. It has the potential to cause either noninvasive, self-limiting, and gastrointestinal infection in healthy individuals or invasive and systemic infection in immune-compromised individuals such as elderly, pregnant women, and new-born by resulting in meningitis, encephalitis, septicemia, mother-to-fetus infection, and abortion [1]. Thus far, six *Listeria* species (*L. monocytogenes*, *Listeria innocua*, *Listeria seeligeri*, *Listeria grayi*, *Listeria welshimeri*, and *Listeria murrayi*) have been well-studied for their public and veterinary health importance. Among these, *L. monocytogenes* is

a predominant food-borne pathogen with emerging antibiotic resistance patterns [2].

The first antibiotic-resistant *L. monocytogenes* strain was identified in France in 1988 and since then many more resistant strains have been isolated from foods and human sporadic listeriosis cases due to excessive use of antimicrobials and disinfectants in the food industry [3, 4]. Pathogenicity of *L. monocytogenes* is determined by several virulence factors [5] such as internalins (*inlA*, *inlB*, *inlC*, and *inlJ*), listeriolysin O (*hlyA*), actin (*actA*), phosphatidylinositol phospholipase C (*PI-PLC*, *plcA*), invasion associated protein (*iap*), and virulence regulator (*prfA*). Detection of more than one virulence-associated genes by PCR technique

has been suggested as an important tool to identify *L. monocytogenes*.

*L. monocytogenes* causes severe infection and accounts for 19% of the total deaths from the major food-borne pathogens [6]. In animals, the clinical manifestation of listeriosis is septicemia, abortion, and latent infection, particularly in ruminants, monogastric animals, and poultry [7]. In humans, the prevalence of listeriosis in the United States has caused 260 deaths annually out of 1,600 infections and is responsible for the highest hospitalization rate (91%) [8]. In the European Union, a total of 1,763 confirmed human cases of listeriosis were reported in 2013, with a fatality rate of 15.6% [9]. In China, 253 invasive listeriosis cases were reported between 2011 and 2016 [10]. A massive listeriosis outbreak in Canada in 1981 provided an initial evidence of *L. monocytogenes* transmission as a food-borne pathogen [11]. Afterwards, the incidence of listeriosis is sharply increasing worldwide, despite antibiotic treatments. In Ethiopia, the prevalence of *Listeria* species (25%) and *L. monocytogenes* (6.25%) has been reported from 384 food samples examined using microbiological methods [12]. Another study in Addis Ababa city indicated that out of 391 food samples of animal origin, 102 (26.1%) were positive for *Listeria* species using microbial culture methods [13]. In Jimma town, from a total of 200 food samples (milk, cottage cheese, ice cream, and yogurt) examined, *Listeria* spp. were detected in 13 (6.5%) of them [14]. According to Welekidan et al. [15], the prevalence of *L. monocytogenes* in pregnant women is 8.5%, with a high prevalence in the age group of 20–24 years (18.6%), housewives (11.4%), rural dwellers (10%), and secondary school students (9.6%). Another study by Girma and Abebe [16] in Debre Birhan town indicated that out of 407 raw milk samples, 85 of them were found positive for *Listeria* species.

*L. monocytogenes* mainly infects warm-blooded ruminants and causes significant economic losses. Furthermore, ruminants may serve as a source of infection for humans, primarily from consuming contaminated animal products [17]. To this effect, rapid, specific, cost-effective, and accurate detection of the microbe is crucial. So far, in Ethiopia, isolation and characterization of *L. monocytogenes* were predominantly carried out using basic microbiological methods. By and large conventional microbes' characterization methods may not discriminate between species or strains within the same species and pathogenic from non-pathogenic strains [18]. Consequently, there is an urgent need for molecular methods that discriminate between species or strains within the same species.

In the southern part of Ethiopia, there is inadequate information regarding *L. monocytogenes* prevalence and its molecular detection in both veterinary and public health sectors. Generally, this pathogen has not yet been identified as a common disease in humans in the study sites. Accordingly, to address this limitation and to fill the existing knowledge gap, advanced molecular-based detection technique is deemed important. Therefore, this study aimed to isolate and characterize *L. monocytogenes* from raw fresh cow milk using the standard microbiological and multiplex

PCR methods. In addition, antibiotics susceptibility profiles of *L. monocytogenes* were determined.

## 2. Materials and Methods

**2.1. Study Area and Period.** The study was conducted from December 2019 to September 2020 at the Molecular Biotechnology laboratory of the School of Animal and Range Sciences, College of Agriculture, Hawassa University, Ethiopia. The study was undertaken in purposively selected two districts (Dale and Arsi Negele) and Hawassa city in southern Ethiopia based on their high potential for milk production (Figure 1). Dale district is about 320 km south of Addis Ababa and 45 km from Hawassa city, the capital of the Sidama Regional State. The altitude of Dale district extends up to 2,600 meters above sea level. The average annual rainfall and temperature vary between 800–1300 mm and 18–20°C, respectively. Arsi Negele district is located in the West Arsi Zone of Oromia Regional State and 225 km away from Addis Ababa, with an altitude that ranges from 1,500 to 2,300 meters above sea level. The total population of the district in 2007 was estimated to be around 264,314 (211,985 rural and 52,329 urban residents). Hawassa is the capital city of Sidama Regional State, located 275 km south of Addis Ababa and located at an altitude of 1,750 meters above sea level. Hawassa receives an average annual rainfall of 955 mm with a mean annual temperature of 20°C. Milk and milk products are important economic activities in Hawassa city and there are a growing number of commercial dairy farms in the area. The city is divided into eight subcities and 32 *kebeles* (20 urban and 12 rural). *Kebele* refers to the smallest administrative cell according to the administration policy of the country.

**2.2. Study Design and Sample Size Determination.** A cross-sectional study design was employed to determine the prevalence of *Listeria* isolates and to characterize *L. monocytogenes* from raw cow milk in the study area. A total of 16 *kebeles* were selected from Hawassa city ( $n = 8$ ), Dale district ( $n = 5$ ), and Arsi Negele district ( $n = 3$ ) based on their potential for milk production. From each *kebele*, smallholder dairy producers having two to five milking cows were selected purposively. Commercial dairy production farms were categorized into three groups based on the number of milking dairy cows that included small (2–5), medium (6–10), and large (>10), as suggested by Ike [19]. Accordingly, from all the study areas, 26 small-size, 23 medium-size, and 51 large-size commercial dairy production farms were randomly selected for the study.

The sample size was estimated based on the preliminary investigation conducted in Hawassa, Ethiopia [20], where the prevalence of *L. monocytogenes* in milk was reported to be 19.55%. Accordingly, 100 samples from commercial dairy production farms and 140 from smallholder dairy producers with a total of 240 raw milk samples were analyzed. From the total, the sample size was distributed to the study areas based on proportion to the number of dairy production farms and smallholder dairy producers.

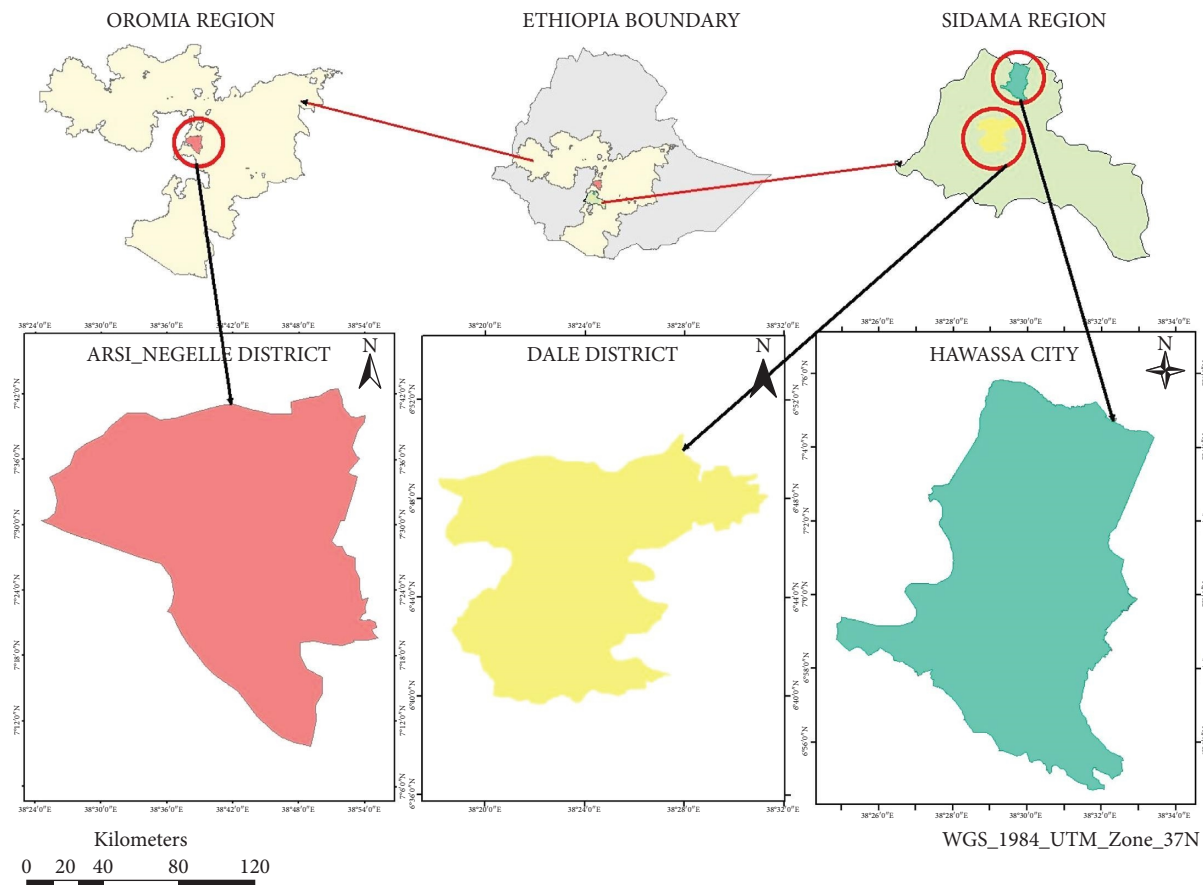


FIGURE 1: Map of the sampling area.

**2.3. Sampling Methods and Collection.** From milking dairy cows, bulk raw milk samples were collected using a simple random sampling technique from the purposively set sampling frame. Samples were collected in two distinct seasons (dry: December–February 2019/20 and wet: July–September 2020) to determine variation due to seasonal effects [21]. From each season, 120 milk samples were collected. Fifty milliliters of raw milk samples were collected aseptically in sterile screw-capped plastic containers from commercial dairy production farms and smallholder dairy producers. After labeling, all the collected milk samples were placed in an icebox and immediately transported to Hawassa University, College of Agriculture, Molecular Biotechnology laboratory within an hour. The samples were stored at 4°C for subsequent activities.

**2.4. Isolation and Identification of *Listeria* Species.** Isolation and identification of *L. monocytogenes* from raw bulk cow milk were carried out following the standard methods [22] with some modifications. This method had a two-stage enrichment process. Briefly, in the primary nonselective enrichment, a 25 ml raw milk sample was measured and transferred into 225 ml of the buffered peptone water (HI media M614, India). In secondary selective

enrichment, 1 ml of homogenized sample from primary enrichment was added to 9 ml of the *Listeria* enrichment broth (Twin pack, HI media M569, India) and incubated at 37°C for 48 h. After the successive enrichment step, 0.1 ml selective enrichment broth culture was streaked using a sterile inoculating loop onto the *Listeria* selective agar (Twin pack, HI media M567, India) plates and incubated at 37°C for 48 h. The plates were observed for the presence of colonies presumed to be *Listeria* spp. For confirmation of *Listeria* species, from each plate of selective medium (LSA, twin pack), five colonies presumed to be *Listeria* spp. were picked and streaked onto the surfaces of predried plates of tryptone soya agar (TSA, HI media M1968, India). The plates were incubated at 37°C for 18 to 24 h. Typical colonies of 1 to 2 mm in diameter, convex shape, colorless, and opaque with an entire edge were considered as presumptive *Listeria* spp. All the isolates from TSA were subjected to further morphological (Gram staining, motility) and standard biochemical (hemolysis, catalase, Esculin hydrolysis, Methyl-red, Voges-Proskauer (MR-VP) reaction and Christie–Atkins–Munch–Peterson (CAMP)) tests. In addition, rhamnose, mannitol, glucose, sucrose, maltose, lactose, and xylose fermentation tests were evaluated and the production of acid indicated positive reactions by turning the media to a yellow color within 24 to 48 h.

## 2.5. Detection of 16S rRNA Region of *Listeria* Isolates and Virulence-Associated Genes

**2.5.1. Genomic DNA Extraction.** Genomic DNA was extracted using the boiling and snap-chilling methods [23]. Suspected colonies of *L. monocytogenes* ( $n = 17$ ) from TSA plates were inoculated into 5 ml tryptone soya broth and incubated overnight at 37°C for 18 h. Overnight enriched bacterial culture (2 ml) from tryptone soy broth was transferred into a 2 ml Eppendorf tube. The broth culture was pelleted by centrifugation at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 100  $\mu$ l of sterile nuclease-free water. The contents were boiled at 100°C for about 10 minutes in a heat block and immediately chilled in an ice cube for about 20 minutes. Finally, it was centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was collected and stored at -20°C for further use as template DNA.

**2.5.2. Amplification of 16S rRNA Region of *Listeria* and Virulence-Associated Genes.** The multiplex PCR protocol was employed for the detection of 16S rRNA-specific genes of *Listeria* species [24, 25] to confirm the genus in all the tested isolates and virulence-associated genes using the standard *L. monocytogenes* (ATCC 11911) as positive control and sterile nuclease-free water as a negative control. The target genes, oligonucleotide primers (Eurofins Genomics, USA), and expected product sizes are listed in Table 1. The mPCR reaction mixture was optimized as 2.5  $\mu$ l of 10x PCR buffer with 15 mM MgCl<sub>2</sub>, 10 mM dNTPs mix (1.0  $\mu$ l), FIREPol DNA polymerase (1.0  $\mu$ l), 0.5  $\mu$ l of 20 pM each forward and reverse primers (16S rRNA, hlyA, plcA, prfA, iapA, and actA), 12.5  $\mu$ l of sterile nuclease-free water, and 2.0  $\mu$ l of DNA template. The DNA amplification reaction was performed in a Thermocycler (UNO<sup>96</sup> Gradient, Germany), with a preheated lid. The cycling condition was one cycle of initial denaturation of DNA at 94°C for 2 min, followed by 35 cycles of amplification (each cycle consisted of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and initial elongation at 72°C for 1 min), and one cycle of a final extension at 72°C for 10 min and held at 4°C. Five microliters of the amplified products were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide and visualized under UV illumination.

**2.6. Antimicrobial Susceptibility Test of *L. monocytogenes*.** A standard Kirby-Bauer disk diffusion method was employed [26] using ten conventional antibiotic discs with the following concentrations: ampicillin, 25  $\mu$ g; chloramphenicol, 30  $\mu$ g; erythromycin, 15  $\mu$ g; gentamicin, 10  $\mu$ g; streptomycin, 10  $\mu$ g; vancomycin, 30  $\mu$ g; nalidixic acid, 30  $\mu$ g; cefotaxime, 5  $\mu$ g; sulfamethoxazole, 25  $\mu$ g; and kanamycin, 30  $\mu$ g. The commonly used antibiotics in veterinary and human therapies with good accessibility to the local market were selected for this study. Three to five colonies of the confirmed *L. monocytogenes* ( $n = 17$ ) from the tryptone soya agar plate were separately transferred into 5 ml physiological saline (0.85%) and adjusted to 0.5 McFarland

turbidity standards ( $1.5 \times 10^8$  cfu/ml). A sterile swab was dipped into the suspension and seeded uniformly onto surfaces of presolidified Mueller Hinton Agar (MHA, M173, HI media, India). Thereafter, the plates were dried, and antibiotic discs were aseptically placed using sterile forceps. The plates were incubated overnight at 37°C. Eventually, the zone of inhibition was measured and the susceptible, intermediate, and resistant categories were assigned and interpreted according to the Clinical and Laboratory Standards Institute [26].

**2.7. Quality Control.** *L. monocytogenes* (ATCC 11911) was used as a reference strain in all the stages of analysis (enrichment, plating, confirmatory tests, and antimicrobial susceptibility). *Staphylococcus aureus* (25923) was used for the CAMP test. The standard strains of *L. monocytogenes* (ATCC 11911) and *S. aureus* (25923) were obtained from the Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia. The reaction mixture without a DNA template was used as a negative control in mPCR.

**2.8. Data Analysis.** Data were analyzed using Statistical Packages for Social Science SPSS 2007, version 20 software. Descriptive statistics was employed to describe prevalence based on location, season, and farm size. The Chi-square ( $\chi^2$ ) was used to determine significant differences among the variables. A probability value of less than 0.05 ( $p < 0.05$ ) was considered statistically significant.

## 3. Results

Out of 240 samples analyzed, 69 (28.75%) were positive for *Listeria* species. Among the 69 (28.75%) positive samples of *Listeria* species, 17 (7.08%) isolates were confirmed to be *L. monocytogenes* after detailed morphological and biochemical tests were conducted (Table 2). The remaining isolates were designated as other *Listeria* species.

The highest and the lowest prevalence of *L. monocytogenes* were isolated from the Arsi Negele (9.41%) and Dale districts (5.56%), respectively. On the other hand, in Hawassa City, 6.02% of the raw milk samples were contaminated with *L. monocytogenes* (Table 2). There was no significant ( $p > 0.05$ ) difference in *L. monocytogenes* isolation rate among locations (Table 2).

The isolates that showed characteristics of Gram-positive, *coccobacillus*, or short rod-shaped were suspected as *L. monocytogenes*. The presumptively identified isolates were further subjected to detailed biochemical tests and hemolysis on sheep blood agar and confirmed as *L. monocytogenes* (Table 3).

The study showed that raw cow bulk milk samples were highly contaminated with *Listeria* species in the wet season, with a detection rate of 36.4% compared to the dry season (21.3%; Table 4). In the study areas, there was a significant ( $p < 0.05$ ) difference in the prevalence of *Listeria* isolates between wet and dry seasons (Table 4). The prevalence of *L. monocytogenes* in the wet season was 9.32% but 4.92% in the dry season (Table 4).

TABLE 1: Primer sequences used in the mPCR reactions for the detection of 16S rRNA and virulence-associated genes in *L. monocytogenes*.

Target gene	Primer sequences (5'-3')	Amplicon size (bp)
16S rRNA	F: CAGCAGCCGCGGTAATAC R: CTCCATAAAGGTGACCCT	938
hlyA	F: GCAGTTGCAAGCGCTTGGAGTGAA R: GCAACGTATCCTCCAGAGTGATCG	456
PlcA	F: CTGCTTGAGCGTTCATGTCTCATCCCC R: CATGGGTTTCACTCTCCTTCTAC	1484
actA	F: CGCCGCGGAAAATTAATAAAGA R: ACGAAGGAACCGGGCTGCTAG	839
prfA	F: CTGTTGGAGCTTCTTGGTGAAGCAATCG R: AGCAACCTCGGTACCATATACTAACTC	1060
iapA	F: ACAAGCTGCACCTGTTGAG R: TGACAGCGTGTAGTAGCA	131

F: forward; R: reverse.

TABLE 2: Prevalence of *L. monocytogenes* in raw milk samples collected from southern Ethiopia.

Location/study areas	No. of samples examined	No. of positive samples for <i>L. monocytogenes</i> (%)	$\chi^2$ ( <i>p</i> value)
Hawassa	83	5 (6.02)	1.35 (0.57)
Dale	72	4 (5.56)	—
Arsi Negele	85	8 (9.41)	1.392 (0.58)
Total	240	17 (7.08)	

TABLE 3: Characterization of *L. monocytogenes* based on biochemical tests.

Biochemical tests	C	M	EH	MR	VP	D-Xy	L-Rh	L	G	S	M	D-Ma	H	Cs
Test reaction	+	+	+	+	+	—	+	V	+	+	+	—	+	+

C, catalase; M, motility; EH, esculin hydrolysis; MR, methyl red; VP, Voges-Proskauer's; D-Xy, xylose; L-Rh, rhamnose; L, lactose; G, glucose; S, sucrose; M, maltose; D-Ma, mannitol; H, hemolysis on 5% sheep blood agar; Cs, CAMP (*S. aureus*); V, variable; +, positive; —, negative.

TABLE 4: Seasonal variations in the prevalence of *Listeria* species isolates, Southern Ethiopia.

Season	N	PS1		$\chi^2$	<i>p</i> value	PS2		$\chi^2$	<i>p</i> value
		NI	%			NI	%		
Dry	122	26	21.3			6	4.92		
Wet	118	43	36.4	6.70	0.01	11	9.32	1.77	0.18
Total	240	69	28.75			17	7.08		

N = number of samples examined; NI = number of positive isolates; PS1 = positive isolates of *Listeria* species including *L. monocytogenes*; PS2 = positive isolates of *L. monocytogenes*.

In this study, both pathogenic and nonpathogenic *Listeria* species were isolated from raw milk samples of smallholder dairy producers and commercial dairy production farms (Table 5). *L. monocytogenes* was isolated from 8.57% raw milk samples of smallholder dairy producers and 5.0% samples of commercial dairy production farms (Table 5). On the other hand, 33 (23.57%) and 19 (19.0%) of other *Listeria* species were isolated from raw milk samples of smallholder dairy producers and commercial dairy production farms, respectively.

*L. innocua* (16.25%) was the most commonly isolated species, followed by *L. monocytogenes* (7.08%), *L. seeligeri* (3.33%), *L. welshimeri* (1.67%), and *L. grayi* (0.42%) in commercial dairy production farms and smallholder dairy producers (Table 6). *L. monocytogenes* was isolated from four (16.67%) raw bulk milk samples of small-scale commercial dairy production farms and 1 (4.54%) sample of medium-scale commercial farms (Table 6). No *L. monocytogenes* was found in samples from large-scale commercial dairy production farms.

TABLE 5: Prevalence of *L. monocytogenes* and other *Listeria* species from raw milk samples of commercial dairy production farms and smallholder dairy producers.

Sources of raw milk samples	No. of samples screened	No. of samples positive for <i>L. monocytogenes</i> (%)	No. of samples positive for other <i>Listeria</i> species (%)	Total positive (%)	$\chi^2$ ( <i>p</i> value)
Smallholder dairy producers	140	12 (8.57)	33 (23.57)	45 (32.14)	1.89 (0.17)
Commercial dairy production farms	100	5 (5.0)	19 (19.0)	24 (24)	
Total	240	17 (7.08)	52 (21.67)	69 (28.75)	

TABLE 6: Percentages of *Listeria* species isolated from raw milk of commercial dairy production farms and smallholder dairy producers.

Types of species isolated	Smallholder dairy producers	Commercial dairy production farms				Total isolated species	Total percent (%)
		SS	MS	LS	%		
<i>L. monocytogenes</i>	12	4	1	—	5.49	17	7.08
<i>L. innocua</i>	23	8	5	3	17.58	39	16.25
<i>L. seeligeri</i>	5	3	—	—	3.30	8	3.33
<i>L. welshimeri</i>	4	—	—	—	—	4	1.67
<i>L. grayi</i>	1	—	—	—	—	1	0.42

SS, small scale; MS, medium scale; LS, large scale.

**3.1. Detection of 16S rRNA and Virulence-Associated Gene Profiles of *L. monocytogenes*.** Analysis of multiplex PCR used for the detection of 16S rRNA and virulence-associated genes using specific primers revealed that of the 17 *L. monocytogenes* isolates, a gene for *Listeria* specific 16S rRNA was found in all the 17 tested isolates, but hlyA and iapA were found only in the 11 tested isolates. On the other hand, no isolate was found to contain prfA, actA, and plcA genes (Figure 2). The virulent-associated genes of iap and hly genes were found in the amplification of 11 tested isolates, but no isolate was found to contain actA, prfA, and plcA genes (Figure 2).

**3.2. Antimicrobial Susceptibility Profiles of *L. monocytogenes*.** Of the total 17 *L. monocytogenes* tested, all (100%) of them were resistant to nalidixic acid, 15 (88.24%) isolates to erythromycin, 4 (23.53%) isolates to ampicillin, 3 (17.65%) isolates to chloramphenicol, 2 (11.76%) isolates to streptomycin, and 1 (5.88%) isolate to cefotaxime (Figure 3). However, all the *L. monocytogenes* isolates ( $n = 17$ ) were sensitive to vancomycin, gentamicin, and sulfamethoxazole.

From 17 *L. monocytogenes* isolates, 16 were found to be multidrug resistant (MDR) against two to three antimicrobial drugs in 5 combinations (Table 7). Eight *L. monocytogenes* isolates were resistant to erythromycin and nalidixic acid, followed by three isolates to ampicillin, erythromycin, and nalidixic acid, two isolates to streptomycin, erythromycin, and nalidixic acid, two isolates to chloramphenicol, erythromycin, and nalidixic acid, and one isolate resistant to chloramphenicol, ampicillin, and nalidixic acid (Table 7).

## 4. Discussion

In the current study, the prevalence of *Listeria* species in raw cow milk samples was higher, particularly in smallholder dairy producers. Other studies in Ethiopia have reported the prevalence of *Listeria* species in foods of animal origin including raw milk [12, 13, 16, 27, 28]. The overall prevalence (28.75%) for *Listeria* spp. reported in this study is similar to the prevalence rate reported by Seyoum et al. [27] from raw cow milk and dairy products in the central highlands of Ethiopia. In the present study, out of the 240 raw milk samples analyzed, *L. monocytogenes* was detected in 17 (7.08%) samples. Similarly, Girma and Abebe [16] and Marnissi et al. [29] have also reported 8.84% and 8% prevalence of *L. monocytogenes* in raw milk samples, respectively. In contrast, Garedew et al. [12] have reported a lower rate of 4.0% in raw milk. A higher rate of contamination in the present study might be due to storage of raw milk at ambient temperature for an extended period of time. Generally, contamination can occur during milking, processing, and storage that poses high public health concern since most of the milk and milk products in Ethiopia are consumed in raw forms without being treated with sufficient heat. Similarly, Sharma et al. [30] have also recovered 25 (21.7%) isolates of *L. monocytogenes* from raw cattle milk samples, while Shrinithiviahshini et al. [31] have also found more (60.6%) positive samples for *L. monocytogenes* in raw milk. These values are higher than the findings reported in the present study that could be associated with several factors such as methods used in pathogen detection, the hygienic status of milk cows, and other parameters. However, this does not mean that milk samples in our study were

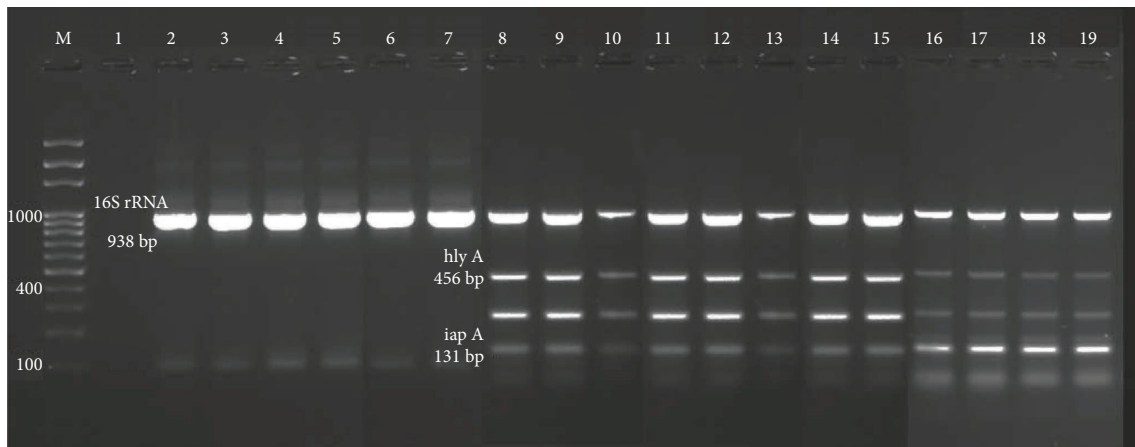


FIGURE 2: PCR detection of *Listeria* from raw milk samples. Lanes 1–7 contain the *Listeria* specific 16S rRNA gene without having the virulent genes, whereas lanes 8 to 18 contain the hlyA and iapA virulent-associated genes with the amplicon size of 456 bp and 131 bp, respectively. Lane M, DNA molecular weight marker (100 bp); Lane 1, negative control; Lane 19, positive control.

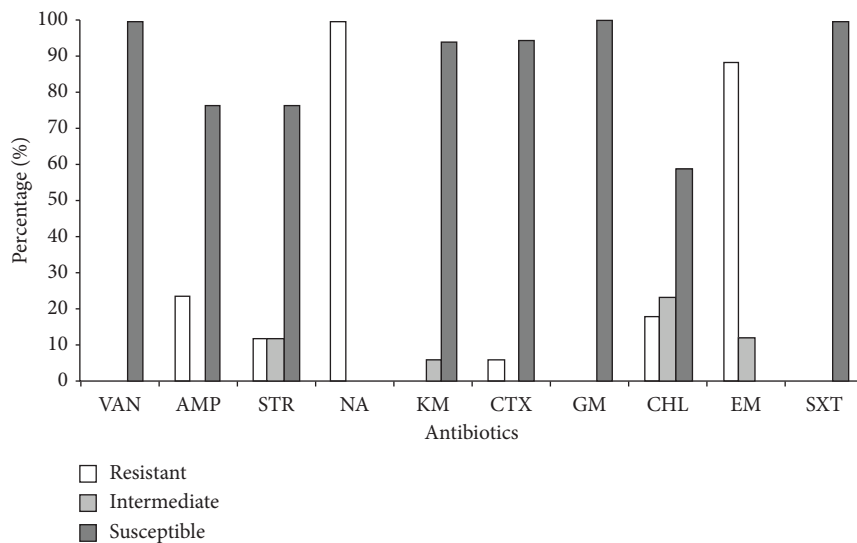


FIGURE 3: Antimicrobial resistance profiles of *L. monocytogenes* isolated from milk samples. VAN: vancomycin; AMP: ampicillin; STR: streptomycin; NA: nalidixic acid; KM: kanamycin; CTX: cefotaxime; GM: gentamicin; CHL: chloramphenicol; EM: erythromycin; SXT: sulfamethoxazole.

TABLE 7: Patterns of multidrug-resistance of *L. monocytogenes* isolated from raw cow milk.

No. of isolates (%)	No. of classes with resistance	Resistance to antimicrobial profile	Classes with resistance
8 (40.05)	2	EM, NA	Mac-Qui
3 (17.65)	3	AMP, EM, NA	Pen-Mac-Qui
2 (11.76)		STR, EM, NA	Ami-Mac-Qui
2 (11.76)		CHL, EM, NA	Chl-Mac-Qui
1 (5.88)		CHL, AMP, NA	Chl-Pen-Qui

AMP: ampicillin; STR: streptomycin; NA: nalidixic acid; CHL: chloramphenicol; EM: erythromycin; Qui: quinolones; Mac: macrolides; Pen: penicillins; Ami: aminoglycosides; Chl: chloramphenicol.

safe to consume. The predominant bacterium isolated in the present study was *L. innocua* (16.25%), which is almost similar to the findings in other parts of Ethiopia [12, 13, 28]. It was also known to be the highest prevalent *Listeria* species elsewhere in the world [3].

Out of 140 raw milk samples of smallholder dairy producers, 32.14% was found to be positive for *Listeria* species that is higher than the prevalence (20.3%) reported by Girma and Abebe [16] for *Listeria* species from raw cow milk samples collected from smallholder producers. This discrepancy might be justified as either the difference in sample size or hygienic practices or methods of detecting the pathogenic species.

In the current study, the highest occurrence (9.41%) of *L. monocytogenes* isolates was obtained from Arsi Negele followed by Hawassa city (6.02%). Such differences among different locations may indicate diverse risk factors associated with the contamination of milk among the three study sites. Discrepancies among the study sites could be attributed to hygienic status of temporary cattle confinement places (barn), hygienic handling practices of milk, milk containers, and milk storage conditions [32]. The differences might also be due to poor hygiene and sanitation practices in milk production, processing, and storage. In Hawassa city, *L. monocytogenes* was isolated from 6.02% of milk samples which might be because of better hygienic practice and health information in the city than the district of Arsi Negele. However, the presence of a single pathogenic cell is equally important since it poses serious public health risk and needs strict intervention measures by the responsible bodies.

The prevalence of *L. monocytogenes* in the present study of small- and medium-scale commercial farms was 15.38% and 4.35%, respectively, but no *L. monocytogenes* was isolated from large-scale commercial farms. This low prevalence of *L. monocytogenes* in large-scale commercial dairy production farms might be due to better management and hygienic handling of raw milk. Although *L. monocytogenes* was not isolated from large-scale commercial farms, 3 (5.88%) of *L. innocua* were isolated, which may reveal possible contamination risks.

Previous studies on *Listeria* spp. prevalence in raw milk reported some evidence of seasonal variation where it was markedly higher during the wet season than during the dry season [33]. This finding was similar to the current study since a higher (9.32%) prevalence of *L. monocytogenes* was observed during the wet season compared to the dry season (4.92%). The sources of *Listeria* spp. in raw milk have been reported to be fecal and environmental contaminations during milking, storage, transport, infected animals in dairy farms, and poor silage quality [34]. However, silage is not widely used as animal feed in the areas where the present study was conducted. Therefore, the higher contamination source of *L. monocytogenes* in raw milk in the wet season is likely due in part to insufficient hygiene during milking, storage, feeding practice, or environmental contamination.

As previously reported [24, 35], all of the morphologically and biochemically described *L. monocytogenes* isolates ( $n = 17$ ) had 16S rRNA genes of the genus *Listeria*. In this

study, virulence-associated genes *iapA* and *hlyA* were discovered in 11 isolates. However, *actA*, *prfA*, and *plcA* genes were not found in any of the isolates. These findings are concurrent with the findings of Rawool et al. [36] who also reported the *hlyA* and *iapA* genes in raw bovine milk. Yadav et al. [37] isolated *L. monocytogenes* from cow mastitic milk samples and used PCR to validate the presence of the *hlyA* and *iapA* genes. Raorane et al. [38] on the other hand have documented all of the virulence genes (*hlyA*, *iapA*, *actA*, *prfA*, and *plcA* genes) from the milk samples analyzed for virulent genes. The absence of one or more virulence determinant genes due to spontaneous mutations in some *L. monocytogenes* strains [39] could explain why only two virulence genes were found in this study. In the current study, none of the *L. monocytogenes* isolates were tested positive for *actA*, *prfA*, and *plcA* genes, which is in line with the findings of Usman et al. [40] who did not detect *actA* or *prfA* genes in milk and milk products but only 20% of *plcA* gene detection was reported by Coroneo et al. [41]. *Listeriolysin O* and *actA*, which are related to the bacterium's capacity to breach the intestinal barrier, cell to cell spread and motility, cell invasion, and intracellular parasitism [42], could be the cause of this variation. As a result, large-scale simultaneous identification of virulence-associated genes should be required since it saves time and effort and is useful in large-scale surveys for detecting the virulent strains of *Listeria* species [36].

The susceptibility patterns of *L. monocytogenes* in the present study revealed 100% sensitivity to the commonly used antibiotic vancomycin as indicated in the previous investigations [12, 16]. Morobe et al. [43] have also reported the susceptibility of *Listeria* species to gentamicin similar to the present study. In contrast, Wong et al. [44] have reported extremely low susceptibility to kanamycin which varies from the finding of the present study (94.12%). Variation in the geographical region and differences in therapeutic practices can be the major contributing factors.

All the tested *L. monocytogenes* showed 100% resistance to nalidixic acid as reported in the previous studies [45, 46]. In contrast, Girma and Abebe [16] and Garedew et al. [12] from Ethiopia, have reported 30.5% and 50% resistance, respectively, to nalidixic acid. These results might be in part due to the excessive use of this antibiotic in dairy cattle production. Other factors that may contribute to the evolution of antibiotic resistance include runoffs from farms, faeces from animals, and workers in milking lines who carry antibiotic-resistant microorganisms [47]. The record of 23.53% resistance to ampicillin in the present study is similar to the findings of Rahimi et al. [48]. On the contrary, a study conducted by Abdollahzadeh et al. [49] showed higher resistance to ampicillin from milk and dairy products in Iran. Likewise, Ennaji et al. [46] have reported that *L. monocytogenes* was 100% resistant to cefotaxime, which is not in agreement with the current study (5.88%).

The presence of antimicrobial-resistant *L. monocytogenes* in raw food products has an important public health implication especially in developing countries where there is widespread and uncontrolled use of antimicrobials [31]. Since the consumption of raw milk is very common, particularly in southern



Ethiopia, the significance of the problem might be higher. The increased antimicrobial administration to livestock could also cause the development of resistance to antimicrobial agents by *L. monocytogenes* [50].

Multidrug resistance of *L. monocytogenes* isolates to erythromycin, ampicillin, and chloramphenicol (this study) is similar to the studies by Al-Ashmawy et al. [50] and Magiorakos et al. [51] who found that the majority of *L. monocytogenes* isolates were resistant to two or more drugs. Furthermore, erythromycin resistance was found in 88.24% of the *L. monocytogenes* species examined, which could have resulted from the overuse of this antibiotic due to its relatively low cost and widespread availability to the local community. Erythromycin is one of the most commonly given medications for most infectious diseases in the current study locations, both in veterinary and human medicine, which could be one of the reasons for the development of such a high resistance profile.

## 5. Conclusion

The finding of this study has shown the presence of antibiotics resistant and virulent *L. monocytogenes* in raw fresh bovine milk collected from southern Ethiopia as revealed using biochemical tests and multiplex PCR methods. These indicate the need to practice adequate heat treatment of raw cow milk before consumption and rational use of veterinary antimicrobial drugs with regular surveillance of antimicrobial resistance to combat multidrug resistance.

One of the limitations of this study was that it did not cover the awareness of commercial farm owners and households towards foodborne pathogens and the examination of clinical samples from aborted animals due to budget and time constraints. Since the study areas are supporting a large number of dairy farms, a comprehensive milk value-chain investigation is required. The prevalence and antibiotic susceptibility patterns of *Listeria* species for meat and other dairy products should be evaluated. Cluster analysis of the isolates should be compared with previously identified species.

## Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethical Approval

The study was ethically cleared and approved by the Graduate Commission of the School of Animal and Range Sciences (SGC 012/19), Hawassa University. Before conducting the research, informed consent was obtained from the owners of the dairy farms included in this study. All methods were carried out in accordance with relevant guidelines and regulations.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

HH designed and performed the experiment, analyzed the data, and prepared the manuscript. MT and DM supervised the research and revised the manuscript. The authors read and approved the final manuscript and agreed to be published.

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