Indian J Dairy Sci 76(6): 542-548
https://doi.org/10.33785/IJDS.2023.v76i06.004

RESEARCH ARTICLE

Listeria monocytogenes isolated from raw milk: phenotypic and molecular characterization, pathogenicity testing, and multidrug resistance profiling

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Received: 20 March 2023 / Accepted: 14 June 2023 / Published online: 23 December 2023

Abstract: Foodborne infections are a worldwide public health emergency that actually impact a variety of disorders. In the current research, a total of 300 samples comprising raw milk (100), milk products (100), and chicken (100) were screened for detection of pathogenic *Listeria* species by using the USDA method. On conventional biochemical characterization, three isolates were identified as *Listeria monocytogenes* (from raw milk samples) indicating an overall prevalence of *Listeria monocytogenes* to the tune of 1%. All other samples comprising milk products and chicken samples (100) showed negativity for the presence of any of the *Listeria* species. Further, all three isolates were subjected to polymerase chain reaction (PCR) targeting genus-specific (*prsA* and *iap*) and species-specific gene (*isp*) in which all three were turn out positive for both the genes, endorsing their identification as *Listeria monocytogenes*. All three confirmed *Listeria monocytogenes* isolates were phenotypically assessed for *in-vitro* pathogenicity tests like hemolysis on 7% sheep blood agar, CAMP test, and PI-PLC assay. The results revealed their highly pathogenic nature. Subsequently, all these isolates were also assessed for their virulent nature by PCR, targeting the array of markers including virulence-associated genes viz. *hlyA, actA, plcA*. The results endorsed pathogenic nature of all isolates showing amplification of all targeted virulence genes. The antibiotic resistance profiling revealed occurrence of multidrug-resistant pathogenic *L. monocytogenes* in foods of animal origin with maximum multiple antibiotic resistance (MAR) index of 0.6 and a minimum MAR index of 0.48 in MDR, which is a matter of concern from public health point of view.

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Keywords: Antibiotic resistance; *hlyA,* Milk; *Listeria monocytogenes*; PCR

Introduction

Listeria monocytogenes is a psychrophilic, Gram-positive, facultative aerobic bacteria and one of the world's most common foodborne diseases (Farber and Peterkin, 1991). The number of species in the genus *Listeria* has increased recently with the discovery of several new ones. There are now 26 species in the genus *Listeria* including *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. marthii, L. costaricensis, L. rocourtiae, L. fleischmannii, L. newyorkensis, L. weihenstephanensis, L. floridensis, L. aquatica, L. thailandensis, L. cornellensis, L. riparia, L. booriae, L. goaensis and L. grandensis, L. valentina, L. farberi, L. portnoyi, L. cossartiae, L. rustica, and L. immobilis*. The most prevalent microbe in both humans and animals among these species is *L. monocytogenes*, while *L. ivanovii* is the one that sickens animals. (Barbuddhe et al. 2022). It leads to severe invasive illness in humans; the main signs are septicemia, abortion, stillbirth, perinatal infections, meningitis, gastroenteritis and meningoencephalitis, particularly in aged and immunocompromised individuals (Posfay-Barbe and Wald, 2004). The incidence of listeriosis caused by this bacterium has skyrocketed in recent years. *Listeria monocytogenes* has been isolated from various foodstuffs, including milk (Barbuddhe et al. 2002), and meat (Lunden et al. 2003 and Bhandare et al. 2007). Typically, this bacterium is found in dairy products like cheese and ice cream manufactured from raw milk (Brooks et al. 2012). In comparison to traditional approaches, molecular techniques like polymerase chain reaction (PCR) provide faster and more reliable results (Borucki et al. 2003). The potential of *Listeria* species to quickly develop resistance to any antimicrobial drug creates a significant and growing hazard to both human and animal health (Luque-Sastre et al. 2018). The current study was designed with the objective to assess the prevalence of vital foodborne pathogen *Listeria monocytogenes* in foods of animal origin viz. milk and meat along with phenotypic and molecular characterization, pathogenicity testing, and multidrug resistance profiling of recovered listerial isolates.

Materials and methods

Sample collection

A total of 300 samples were collected and screened for microbiological evaluation in the current study, including raw milk (100) and milk products (100) from small scale milk vendors and farmers, and chicken (100) from meat shops in the Udgir tehsil of Maharashtra state. Milk product and chicken samples were collected in sterile zip-lock bags while, raw milk samples were collected in sterile milk sampling bottles (35 ml, International Scientific Supplies Ltd., UK).

Bacterial strains

The standard strains of *L. monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 12600), and *Rhodococcus equi* (ATCC 6939) were used in the present study which were obtained from Himedia, Mumbai.

Isolation and Phenotypic characterization of *Listeria* species

Samples were collected aseptically and processed immediately after collection, for the isolation of *Listeria* species as per the protocol suggested by the USDA method as described by Curtis and Lee (1995) with suitable modifications.The protocolincludes two-step enrichment with the University of Vermont (UVM-I and II) and subsequent streaking onto polymyxin-Acriflavin-Lithium chloride Ceftazidime Aesculin-Mannitol (PALCAM) medium as a selective agar. Phenotypically, isolates were characterized by employing a battery of biochemical and sugar fermentation tests. Biochemical testing comprised catalase, oxidase Methyl Red-Voges Proskauer (MR-VP), and nitrate reduction tests, while sugar fermentation tests were carried out with Alpha-Methyl-D-Mannoside, Rhamnose, and Glucose (Dextrose) as per the method described in Cruikshank et al. (1975) and Bergey's Manual of Systematic Bacteriology (1984).

In-vitro pathogenicity testing

In order to assess the pathogenic potential of recovered isolates of *L. monocytogenes* phenotypically, the isolates were subjected to *in-vitro* pathogenicity tests like hemolysis on 7% sheep blood agar (SBA) (Courtieu 1991), Christie, Atkins, Munch-Petersen (CAMP) Test (Christie et al. 1944) and Phosphatidylinositol-specific Phospholipase-C (PI-PLC) assay (Notermans et al. 1991).

PCR confirmation of isolates

The recovered listerial isolates characterized by biochemical tests and sugar fermentation tests were further confirmed by PCR, targeting genus-specific genes (*prsA* and *iap*), and *L. monocytogenes* species-specific (*isp*) gene, adopting the protocol suggested by Rawool, et al. (2016) and Bubert et al. (1992). The PCR conditions and primers used are summarized in Table 1 and 2. The DNA was extracted by using the snap chill method as method described by Rawool et al. (2007). The PCR assay in a final volume of 25 µL was done using the following: 1 × PCR buffer, dNTPs, 50 mM $MgCl_2$, 10 pM of each Primer, HotStar® Taq polymerase (QIAGEN, Hilden, Germany) and standard strain of *L. monocytogenes* MTCC 1143 (Serotype 4b) was used as a positive control. The amplified PCR product was subjected to electrophoresis in a 1.5% agarose gel (in TBE buffer), stained with ethidium bromide (10mg/mL) solution, and finally visualized with a UV transilluminator coupled with a digital gel imaging system (UVP Gel Seq. Software) (Bio-Rad GelDoc Go System, USA).

Moreover, the pathogenic character of confirmed *L. monocytogenes* isolates was also performed by targeting the array of virulence markers viz. *hlyA, actA, plcA* (Rawool et al. 2007) as per the primers and amplification conditions mentioned in Table 1 and 2 and with similar volume and concentration of components as mentioned earlier.

Antimicrobial susceptibility testing

Antimicrobial resistance profiling of recovered isolates was performed by disc diffusion method as per the procedure given by Agarwal(1974). In a nutshell, a single isolated colony of *Listeria* isolates from a Brain Heart Infusion agar plate was grown in Muller Hinton broth for 12-16 hours at 37°C, followed by 1 ml freshly grown culture spread over the Muller Hinton agar plate. Antimicrobial discs were placed at appropriate positions on agar plates and incubated at 37°C. Zones of inhibition were measured after 18 hours and again after 48 hours of incubation using an antibiotic zone scale. The antibiotic susceptibility of recovered *Listeria* isolates was determined based on the data provided in CLSI, (2012). The isolates were tested against a panel of 25 unique antibiotics belonging to different classes and which are routinely used in human and animal treatment as given in Table 3.

Determination of the MAR index

The method given by Osundiya et al. (2013), which divides the number of antibiotics employed in the study by the number of antibiotics an isolate is resistant to (a), was used to calculate the MAR index (b). The following is the calculation formula: Index MAR = a/b.

Results and discussion

Prevalence of *Listeria monocytogenes* in food samples

In this study, on microbiological analysis of 300 food samples comprising raw milk, milk products, and chicken, three presumptive listerial isolates were recovered and identified as *Listeria monocytogenes* on the basis of results of a battery of biochemical tests and molecular detection by PCR, giving an overall occurrence of *Listeria monocytogenes* to the tune of 1%.

The other samples comprising milk products and chicken showed negativity for the presence of any of the *Listeria* species. Amongst these, all three isolates were recovered from raw milk samples revealed a prevalence of 3%.

The prevalence of *Listeria* spp. in milk detected in this study is consistent with the findings of Lovett et al. (1987), who reported a 4.2% overall prevalence of *Listeria* spp., and Gaya et al. (1996), who reported a 2.56% prevalence of *L. monocytogenes* recovered from raw milk samples. Globally, the results of Beak et al. (2000) with 4.4% prevalence from raw milk in Korea, Aygun et al. (2006) with 2.12% prevalence from raw milk in Antakya, Turkey, and Indian data reported by Karthikeyan et al. (2015) and Bhilegaonkar et al. (1997) with 3.5% and 4.9% prevalence *Listeria* spp. in milk, respectively, can be corroborated. These variations of prevalence observed may be attributed to the fact that *Listeria* spp. typically affects raw milk by contamination caused by unhygienic conditions in the environment, gastrointestinal tract, and teat skin of animals.

Furthermore, many researchers, including Aurora et al. (2006), Kalorey et al. (2008), Khan et al. (2013), Shantha and Gopal (2014), Sharma et al. (2017), and Shakuntala et al. (2019), found a 1.69%, 0.1% (2/2060), 0.8%, 0.76%, 1.09%, and 1.7% prevalence of *Listeria* spp. in milk, which were on the low side as that of results of the current study. However, certain studies have reported quite a higher recovery of *Listeria* spp. in milk which includes Mary et al. (2017) with 52.7% (219/415) and Gebretsadik et al. (2011) with 22% (22/100) prevalence. The other factors that also contribute to listerial contamination include lack of hygiene, environmental contamination, and poor milking practices. In this study, raw milk samples were obtained from small scale milk vendors and farmers. It is believed that milk is diluted with water before being sold to customers as a malpractice in order to increase the amount, which could explain why *Listeria* spp. was found in the raw milk sample.

Biochemical characterization and *in-vitro* pathogenicity testing of *Listeria* isolates

On biochemical characterization, all three recovered isolates showed the typical greyish green, glistening, iridescent, and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis on PALCAM agar (Curtis and Lee, 1995), characteristic Gram-positive coccobacilli morphology, tumbling motility in hanging drop technique (Islam et al. 2016) and positivity towards catalase, MR-VP, nitrate

Table 1 Primers and amplicon size for the PCR assays

Target gene	Sequence (5'–3')	Amplicon (bp)	Reference
prs	F=AGCTGAAGAGATTCCGAAAGA R=TTCACCAAGAAGAGCTGCAA	844	Rawool et al. (2016)
iap	F=ACAAGCTGCACCTGTTGCAG R=TGACAGCGTGTGTAGTAGCA	131	Bubert et al. (1992)
isp	F=TGCAGCGAATGCTCTTAGTG R=AGCCAAGCACGGCTACTTTA	713	Rawool et al. (2016)
plcA	F=CTGCTTGAGCGTTCATGTCTCATCCCCC R=CATGGGTTTCACTCTCCTTCTAC	1484	Notermans et al. (1991)
hlyA	F=GCAGTTGCAAGCGCTTGGAGTGAA R=GCAACGTATCCTCCAGAGTGATCG	456	Pazaik-Domanska et al. (1999)
actA	F= CGCCGCGGAAATTAAAAAAAGA R= ACGAAGGAACCGGGCTGCTAG	839	Suarez and Vazquez-Boland (2001)

Table 2 Amplification conditions for the PCR assays

Target gene	Amplification conditions
prs	95°C (5 min), 95°C (30 sec), 53°C (1 min), 72°C (2 min), 72°C (10 min), 40 cycles
iap	95°C (5 min), 95°C (15 sec), 57°C (1 min 20 sec), 72°C (2 min), 72°C (7 min), 40 cycles
isp	95°C (5 min), 95°C (30 sec), 53°C (1 min), 72°C (2 min), 72°C (10 min), 40 cycles
plcA *hlyA* *actA*	95°C (5 min), 94°C (15 sec), 60°C (30 sec), 72°C (1 min 30 sec), 72°C (10 min) , 35 cycles

reduction test and negativity to oxidase test. After confirmation of genus *Listeria*, isolates fermented only the Alpha-Methyl-D-Mannoside, Rhamnose, and Glucose (Dextrose) sugars on sugar fermentation tests, identifying the species of all three isolates as *Listeria monocytogenes* (OIE terrestrial manual 2021 and Nayak et al. 2015). Further, the isolates were judged for their pathogenic potential by employing *in-vitro* pathogenicity tests like hemolysis on SBA, CAMP Test, and PI-PLC assay. In this investigation, all *L. monocytogenes* isolates showed typical beta (β) haemolysis on 7% sheep blood agar and positivity for pathogenicity in CAMP Test and PI-PLC assay revealing their virulent character.

Confirmation of isolates by genus and species-specific PCR

The isolates that exhibit characteristic biochemical and sugar fermentative characteristics were exposed to PCR targeting the *prsA* and *iap* (genus-specific) and *isp* (species-specific) genes, with a product size of 844 bp,131bp, and 713 bp respectively as per results obtained by Rawool, et al. (2016) and Bubert et al. (1992). All 3 listerial isolates showed positivity towards all the three targeted genes, -hence confirming the organisms as *Listeria monocytogenes* (Figure 1 and 2).

PCR targeting virulence-associated genes of *Listeria monocytogenes*

The confirmed *Listeria monocytogenes* isolates were further subjected to PCR that targeted virulence-associated genes to check the virulence character of isolates viz., Actin filament protein (*act*A), haemolysin called listeriolysin O (*hly*A) and invasion associated internalin gene (*Inl*C). The results of the experiment showed that all three isolates (*Listeria monocytogenes*) are positive for all targeted three genes, which were haemolytic on 7% sheep blood agar, CAMP positive, and showed PI-PLC activity, suggesting their pathogenic nature (Figure 3 and 4). The same kind of results was obtained by Rawool et al. (2007) who carried out a PCR employing virulence-associated genes of *L. monocytogenes*, i.e., *plc*A, *hly*A, *act*A, and iap. Also, Arslan and Baytur (2019) targeted *hlyA*, *actA*, *inlA, inlB, inlC, inlJ*, *prfA*, *plcA*, and *iap* virulence-associated genes and reported 100% positivity.

Antibiotic sensitivity test

The inappropriate use of antibiotics for therapeutic purposes in animal and human medicine has led to the development of antibiotic resistance, a major public health issue. Antibiogram study of isolates revealed multiple drug resistance patterns. The panel of 25 different antibiotics along with their different therapeutic concentrations were used, out of which all isolates showed complete resistance (100%) against 12 antibiotics of different groups viz., ampicillin, amoxicillin-clavulanic acid, amoxicillin-sulbactam, cefaclor, cefalexin, cefepime, cefotaxime, furazolidone, oxacillin, streptomycin, sulphadiazine, trimethoprim. The listerial isolates showed 100% sensitivity towards

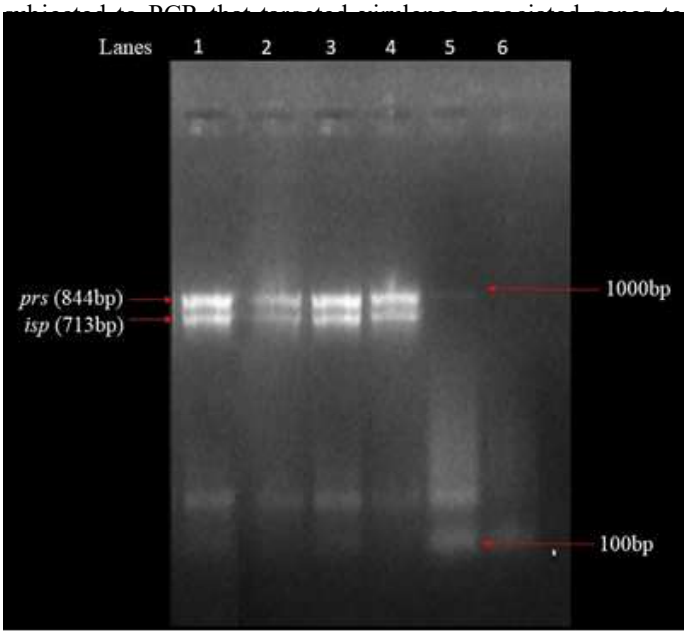

Fig. 1 PCR profile of genus-specific (*prs*) and species- specific (*isp*) genes of *L. monocytogenes*
Lane 1 Positive control template
Lane 2-4 *L. monocytogenes* positive isolates from milk (Both *prs* and *isp* positive)
Lane 5 100 bp DNA ladder (Promega, USA)
Lane 6 NTC (Negative control template)

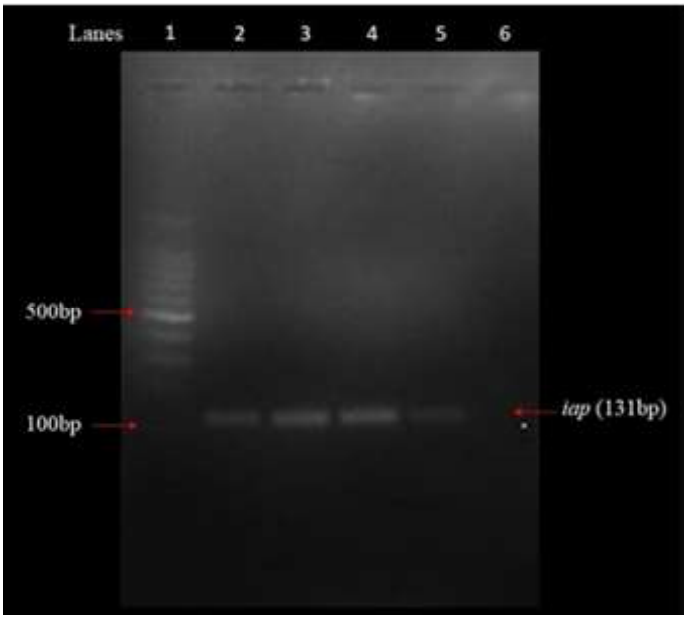

Fig. 2 PCR profile of genus-specific *iap* gene of *L. monocytogenes*
Lane 1 100 bp DNA ladder (Promega, USA)
Lane 2-4 *L. monocytogenes* positive isolates from milk (*iap* positive)
Lane 5 Positive control template
Lane 6 NTC (Negative control template)

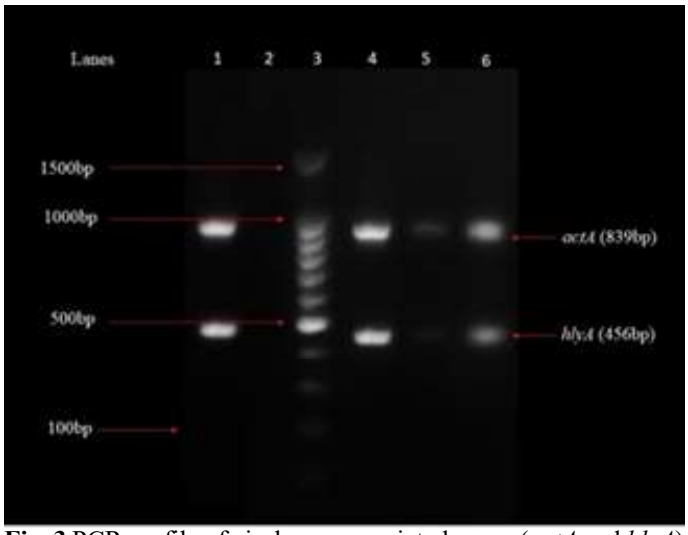

Fig. 3 PCR profile of virulence-associated genes (*actA* and *hlyA*) of *L. monocytogenes*
Lane 1 Positive control template
Lane 2 NTC (Negative control template)
Lane 3 100 bp DNA ladder (Promega, USA)
Lane 4-6 *L. monocytogenes* positive isolates from milk (Both *actA* and *hlyA* positive)

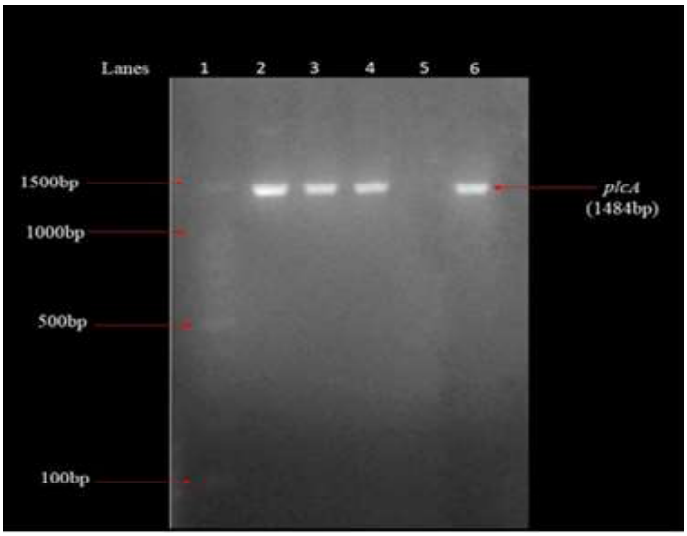

Fig. 4 PCR profile of virulence-associated gene *plcA* of *L. monocytogenes*
Lane 1 100 bp DNA ladder (Promega, USA)
Lane 2-4 *L. monocytogenes* positive isolates from milk (plc*A* positive)
Lane 5 NTC (Negative control template)
Lane 6 Positive control template

Table 3 Antibiogram pattern of *L. monocytogenes* isolates

Sr. No	Antibiotics	Concentration	No. of isolates (n=3)			Percentage (%)		
			S	I	R	S	I	R
1.	Ampicillin (AMP)	10 mcg	0	0	3	0	0	100
2.	Amoxycillin-clavulanic acid (AMC)	25 mcg	0	0	3	0	0	100
3.	Amoxicillin-sulbactam (AMS)	30/15 mcg	0	0	3	0	0	100
4.	Azithromycin (AZM)	10 mcg	3	0	0	100	0	0
5.	Cefaclor (CF)	30 mcg	0	0	3	0	0	100
6.	Cefalexin (CN)	30 mcg	0	0	3	0	0	100
7.	Cefepime (CPM)	30 mcg	0	0	3	0	0	100
8.	Cefotaxime (CTX)	10 mcg	0	0	3	0	0	100
9.	Ciprofloxacin (CIP)	10 mcg	3	0	0	100	0	0
10.	Doxycycline (DO)	10 mcg	3	0	0	100	0	0
11.	Enrofloxacin (EX)	10 mcg	3	0	0	100	0	0
12.	Furazolidone (FR)	50 mcg	0	0	3	0	0	100
13.	Gentamicin (GEN)	50 mcg	3	0	0	100	0	0
14.	Lincomycin (L)	10 mcg	1	1	1	33.33	33.33	33.33
15.	Nalidixic Acid (NA)	30 mcg	3	0	0	100	0	0
16.	Nitrofurantoin (NIT)	200 mcg	2	0	1	66.66	0	33.33
17.	Oxytetracycline (O)	30 mcg	3	0	0	100	0	0
18.	Oxacillin (OX)	5 mcg	0	0	3	0	0	100
19.	Ofloxacin (OF)	2 mcg	3	0	0	100	0	0
20.	Penicillin-G (P)	2 units	3	0	0	100	0	0
21.	Streptomycin (S)	25 mcg	0	0	3	0	0	100
22.	Sulphadiazine (SZ)	100 mcg	0	0	3	0	0	100
23.	Tetracycline (TE)	10 mcg	3	0	0	100	0	0
24.	Trimethoprim (TR)	30 mcg	0	0	3	0	0	100
25.	Vancomycin (VA)	10 mcg	1	2	0	33.33	66.66	0

(S- Sensitive, I-Intermediate, R-Resistant)

Table 4 MAR index isolates

Sr. No.	Origin of isolates	Resistance to antibiotics	Resistance to the number of antibiotics	MAR index
1.	Raw milk	AMS, AMP, AMC, CN, CF, CTX, CPM, S, TR, OX, SZ, FR, VA, L, NIT	15	0.6
2.	Raw milk	AMS, AMP, AMC, CN, CF, CTX, CPM, S, TR, OX, SZ, FR	12	0.48
3.	Raw milk	AMS, AMP, AMC, CN, CF, CTX, CPM, S, TR, OX, SZ, FR, NIT	13	0.52

azithromycin, ciprofloxacin, doxycycline, enrofloxacin, gentamicin, nalidixic acid, oxytetracycline, ofloxacin, penicillin-G, tetracycline, antibiotics as shown in Table 3.

In this research, 100% resistance observed against 12 different antibiotics belonging to different groups can be validated with the results of Prazak et al. (2002) who reported 100% resistance to oxacillin and 85% toward penicillins from food samples, which concerns directly public health as both the antibiotics used as a treatment of listeriosis in combination with the gentamicin (Gomez et al. 2014). Troxler et al. (2000) also stated that all pathogenic *Listeria* species i.e., *L. monocytogenes* and *L. innocua* were naturally resistant to the modern cephalosporins, due to the absence of proper penicillin-binding proteins (PBPs) in the cytoplasmic membrane of *Listeria* which validates our findings. while natural sensitivity towards fluoroquinolones and aminoglycosides also validates our results. The findings of Abdollahzadeh et al. (2016), who also revealed susceptibility showed against tetracyclines by listerial isolates recovered in their research, can be used to support the results observed in present study that listerial isolates were sensitive to tetracyclines.Higher resistance to ampicillin in current research can be related to the work of Soni et al. (2013), who reported 100% resistance towards ampicillin.

MAR index

The multi-drug resistant (MDR) isolates in the present study showed resistance to a minimum of 5 and a maximum of 15 antibiotics. Thus, 3 resistance patterns were observed ranging from 5 to 15 antibiotics with maximum multiple antibiotic resistance (MAR) index of 0.6 and a minimum MAR index of 0.48 in MDR isolates as depicted in Table 4. These findings are comparable with Shourav et al. (2020), who got MAR indices ranging from 0.40 to 0.64 to a panel of 25 antibiotics from recovered *Listeria* isolates. Multidrug resistant *Listeria* were also discovered by Kuan et al. (2017), Swetha et al. (2021), and Elsayed et al. (2022), with MAR indices ranging from 0.11 to 0.56, 0.56 to 0.78, and 0.22 to 0.78, respectively.

Conclusion

To summarise, the current study found 3% prevalence of pathogenic *L. monocytogenes* in milk. Moreover, the listerial isolates revealing pathogenic nature in phenotypic *in-vitro* pathogenic assays were also observed showing the presence of all targeted virulence associated genes viz. *plcA*, *hlyA*, *actA* in PCR, demonstrating excellent correlation between phenotypic and genotypic assays. Further, multidrug-resistant character shown by all pathogenic food borne *L. monocytogenes* isolates are a matter of concern from a public health point of view.

Acknowledgement

The authors are thankful to the Associate Dean, College of Veterinary & Animal Sciences, Udgir for his help and support.

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