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Prevalence, Virulence and Antibiotic Susceptibility of *Listeria monocytogenes* Recuperated from Slaughtered Goats and Pigs of Nagpur, Central India

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ABSTRACT

Listeria monocytogenes is an emerging foodborne pathogen of public health concern. Animals may act an asymptomatic carrier and can contaminate foods of animal origin such as meats and dairy products. The objective of the study was to determine the prevalence, pathogenicity and antimicrobial profile of *L. monocytogenes* in goats and pigs intended for human consumption. A total of 350 samples comprising meat, milk, blood, and faeces of goats and pigs were analysed. Pathogen was isolated employing two step enrichment followed by plating on selective media. Isolates were characterized based on biochemical tests, haemolysis on blood agar, CAMP test, phosphatidyl inositol-specific phospholipase C assay and *hlyA* PCR. Antimicrobial sensitivity was assessed by disc diffusion method. Results of this study revealed 7 (14%) and 10 (20%) prevalence of *L. monocytogenes* in goat and pigs respectively. All the isolates showed in-vitro virulence characteristics such as hemolysis on 5% sheep blood agar, positive CAMP test, PI-PLC activity and presence of *hlyA* gene. Antibiotic sensitivity assay showed that the isolates exhibited variable resistance pattern. Maximum sensitivity was observed for Penicillin-G and Cloxacilin whereas; resistance was noted towards Kanamycin, Rifampicin Cephalexin, Oxytetracycline, Ceftriaxon, Ampicillin, Erythromycin, Norfloxacin and Gentamicin. This study highlights a potential risk of foodborne listeriosis for chevon and pork consumers of this region.

Keywords

Antimicrobial
resistance, Goats,
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Introduction

Listeria monocytogenes is a major food-borne zoonotic bacterium of serious public health concern. Ingestion of contaminated meat and meat products is one of the important causes of foodborne listeriosis in humans. *Listeria* species are most commonly found in raw foods, vegetable contaminated by soil and

water carrying bacteria and in raw animal products such as meat and cheese. Listeriosis accounts for 25-50% death rate among babies born to the women contracting infection during pregnancy (Pal *et al.*, 2017). It was recognised as an important emerging food-borne pathogen by the World Health Organization in the year 1986. The major outbreaks of human listeriosis in the USA, Canada, Western Australia, Europe, France

and Switzerland acknowledged *L. monocytogenes* as a significant food-borne pathogen (Pal *et al.*, 2017). According to an estimate from CDC, 2011 report around 300 deaths were caused by *Listeria* infection each year in the USA. Another news report from Pretoria, South Africa in December, 2017 claimed that food-borne listeriosis accounted for 36 deaths and infection in 600 people (<https://www.businesslive.co.za/bd/national/health/2017-12-05-gauteng-at-centre-of-listeriosis-outbreak>). In India, this pathogen has been isolated from humans, animals and foods with an incidence comparable to those reported elsewhere in the world (Barbuddhe *et al.*, 2012).

In the year 2000, USDA updated the cost-estimates for four pathogens namely *Campylobacter*, *Salmonella*, *E. coli O157:H7*, and *Listeria monocytogenes* based on the CDC's annual foodborne illnesses, and put the total cost in the United States for these four pathogens as \$6.5 billion a year. Of these four pathogens, *Listeria* alone amounted to cost \$2.3 billion per year (Crutchfield *et al.*, 2000). Unfortunately, the burden of food-borne disease in India is not known. Most food-borne diseases go unreported, only few are reported by the media, usually those with high morbidity occurring in urban areas and lack of awareness about the disease makes it difficult to estimate an impact of the listeriosis on the health economy of India.

Ever since the establishment of *Listeria* as a foodborne pathogen, there was growing curiosity in understanding the risk associated with this organism in various sources of food including the foods of animal origin. The majority of meats consumed in India are fish, mutton, chevon, pork, and chicken. Goat meat represents an important foodstuff in nutrition of people especially in China, India, Pakistan and Nigeria (Gefu, 1982). Goat meat is being increasingly consumed in India owing to its

distinctive taste and desirable chemical composition. Whereas, shedding the past poor image, pork is also slowly gaining popularity in India. However, the prevalence status of *Listeria* in these food animals from the region is poorly known. In Nagpur region large mass of consumers prefer chevon and pork, where goats and pigs are being slaughtered at two major slaughter houses in addition to many door-steps of butcher's houses. Considering the facts and the zoonotic potentials of the *Listeria monocytogenes* in foods of animal slaughtered in the region the present study was aimed to determine a prevalence, pathogenicity and antimicrobial profile of *Listeria monocytogenes* in goats and pigs slaughter for human consumption.

Materials and Methods

This study was carried out for a period of one year during 2012-2013.

Study area

Nagpur is a third largest city of Maharashtra geographically located at 79 degree 7 minutes east longitude and 21 degree 7 minute north latitude. It is situated at a height of 312.42 meters above sea level. Nagpur has tropical savannah climate (Koppen climate classification) with dry conditions prevailing for most of the year.

To suffice the food demand of growing human population in Nagpur, foods of animal origin exquisitely supplement agricultural activities. As in other parts of the country, in Nagpur too goats and pigs are reared by the lower section of the society. These animals are mostly managed under extensive and semi-extensive conditions which expose them to the various infectious agents. Moreover, feeding of goats and sheep with corn silage or other silages made from forage and small grain crops which may be poorly fermented or

mouldy put them to the biggest risk of listeriosis. Further, scavenging behaviour of pigs and practices of feeding left over kitchen waste also increases the risk of listeriosis in pigs.

Sample collection

A total of 450 samples comprising 250 of goats and 200 of pigs were collected from Bhandewadi slaughter house, Immamwada slaughter house, goat farm at Nagpur veterinary college and Borgaon region located in Nagpur district (Table 1).

All the samples were collected in sterile test tubes, poly-ethylene sachets, transported on ice to the laboratory and were immediately processed for microbiological analysis.

Isolation and identification

The samples were processed by employing two step enrichment methods. The meat samples weighing approximately 10 gm were aseptically blended by stomacher paddle (Lab Med, UK) in 15 ml of NSS. One ml of homogenized solution; 1ml milk; blood clots after separation of serum and 1gm of faecal samples were transferred to the respective labelled tubes containing 9ml UVM-I broth and incubated for 24 hr at 37°C. After incubation 1 ml turbid inoculum from UVM-I was transferred to 9ml of UVM- II which was further incubated at 37°C for 18- 24 hr.

Selective plating was done by streaking loopful of enriched inoculum from UVM-II onto the PALCAM agar plates and was incubated at 37°C for 48 hr. The presumptive *Listeria* colonies showing typical characteristics were subjected to Gram staining, motility testing, biochemical characterization, *in-vitro* pathogenicity assays and antimicrobial sensitivity test.

Characterization of the isolates

The presumptive *Listeria* colonies were subjected to Gram staining. Gram positive coco-bacillary to rod shaped organisms were inoculated into Brain Heart infusion (BHI) broth and incubated at 25°C for 12 hr to observe characteristic tumbling motility of *L. monocytogenes* under 40X magnification. The isolates with tumbling motility were considered for further biochemical and molecular characterization.

Biochemical characterization

The biochemical characterization was carried out as per the protocol described by Bergey's manual of systematic bacteriology (1984). The isolates were characterized biochemically by catalase, oxidase, MR-VP, nitrate reduction and sugar fermentation (α -methyl D-mannopyranoside, L-rhamnose and D-xylose) tests.

Multiplex PCR for genus and specific confirmation

All the biochemically positive isolates were further confirmed by multiplex PCR targeting genus specific PrsA gene and species specific Isp gene. The reaction was carried out using published primer sets namely, prs-F-TAGCTGAAGAGATTGCGAAAGA and prs-R- CCAAGAAGAGCTGCAACAGATA for PrsA gene and isp-F-TGCAGCGAATGCTCTTAGT and isp-R-CCAAGCACGGCTACTTTAATC for Isp gene. Amplification was carried out in 25 μ L reaction volume in a thermal cycler (Applied Biosystem, USA) with one cycle of initial denaturation at 95°C for 5 min followed by 40 cycles of each denaturation at 95°C for 30 sec, annealing at 53°C for 1 min and extension at 72 °C for 2 min. Finally reaction was concluded with one cycle of final extension at 72°C for 7 min.

Virulence determinants

Haemolysis on sheep blood agar (SBA)

Haemolytic activity of *Listeria monocytogenes* due to production of haemolysin was checked on sheep blood agar (SBA) by streaking freshly grown isolates onto the blood agar plates containing 7% defibrinated sheep blood. The plates were incubated at 37°C for 24 hr and observed for the zone of haemolysis around colonies.

Christie Atkins Munch Petersen (CAMP) Test

Freshly grown isolates of *Staphylococcus aureus* (MTCC 3160) and *Rhodococcus equi* (MTCC 1135) were streaked onto SBA plates (7%) in a manner of straight line with a distance of 3-4cm so that the streaks were wide apart and parallel to each other. Isolates from this study were then streaked in between two parallel streaks of *S. aureus* and *R. equi* at an angle of 90degree leaving a space of 3 mm against parallel streaks. The plates were incubated at 37°C for 24 hr. The isolates with CAMP positivity against *S. aureus* were characterized as *L. monocytogenes* and were designated as pathogenic whereas, those positive against *R. equi* were identified as non-pathogenic *L. ivanovii*.

Phosphatidylinositol-specific phospholipase -C (PI-PLC) assay

Production of phosphatidylinositol-specific phospholipase (PI-PLC) has been reported to be a reliable indicator to discriminate pathogenic and non-pathogenic *Listeria* species. The PI-PLC assay was performed according to Yadav *et al.*, (2010). The isolates of *Listeria* were overgrown on SBA (7%) at 37°C and then streaked onto *L. mono* differential agar (Hi Media Ltd, Mumbai, India) and were incubated at 37°C in a humidified chamber for 24hr. Development of

light blue green color colonies were considered positive for PI-PLC assay and accordingly the isolates were designated as pathogenic and non-pathogenic.

Assessment of hlyA gene using PCR

Listeriolysin O is one of the important virulent markers encoded by *hlyA* gene and is essential for disruption of phagocytic vacuole leading to the release of bacteria in cytoplasm. The specific amplification for *hlyA* gene was carried out using the oligonucleotide sequences *hlyAF*-5'-GCAGTTGCAAGCGCTTGGAGTGAA-3' and *hlyAR*-5'-GCAACGTATCCTCCAGAGTGATCG-3' synthesized from Sigma Aldrich Pvt. Ltd., New Delhi, India. DNA templates for PCR were prepared by simple boiling and snap chilling method as per (Shakuntala *et al.*, 2006). The reaction volume and the cycling conditions for PCR amplification was same as mentioned earlier for multiplex PCR.

Antibiotic sensitivity test

Antibiotic sensitivity of *Listeria* isolates to various antibiotics and chemotherapeutic agents was studied by agar disc diffusion method using single antibiotic disc. The selection of antibiotic was based on the routinely used antibiotic by clinicians in the region (Table 2).

Results and Discussion

Prevalence of *Listeria monocytogenes* among goats and pigs

The observation on screening of 200 goat samples comprising 50 each of chevon, milk, faeces and blood and 150 pig samples comprising 50 each of pork, faeces and blood from Nagpur region revealed seven goat samples (3 chevon and 4 faecal) and ten pig

samples (4 pork and 6 faecal) to be positive for isolation of *Listeria monocytogenes* with overall positivity of 14% (7/50) and 20% (10/50) among the goat and pig populations respectively of Nagpur region. In the present study no two samples from a same animal were recorded positive for *Listeria spp.* Therefore, in the present study, the positivity of *Listeria monocytogenes* was recorded to the tune of 6%, 8%, 8% and 12% in chevon, goat faeces, pork and pig faeces respectively. None of a blood sample of either goat or pig and milk of goat was found positive for *Listeria species* (Table 3).

The observation on cultural characteristics of these isolates recorded aesculin hydrolysis with greenish black pin point colonies on PALCA Magar (Fig. 1). Subsequent Gram staining of isolates revealed all the isolates as Gram positive, coco bacillary rods demonstrating characteristic tumbling motility at 25°C. Further, biochemical analysis revealed catalase positive, oxidase negative, MR-VP positive and nitrate reduction negative for all the isolates.

Sugar fermentation assay confirmed all the isolates as *Listeria monocytogenes* however, some variations in the sugar fermentation pattern was noted. Revalidation of isolates targeting genus specific *PrsA* gene and species specific *isp* gene employing multiplex PCR revealed desired two bands corresponding to 842bp and 711bp respectively confirming the isolates as *Listeria monocytogenes* (Fig. 2).

***In-vitro* pathogenicity tests**

Haemolysin production

In the present study haemolysin production was demonstrated by all 17 isolates of *L. monocytogenes* onto 7 % sheep blood agar. Based on degree (narrow or wider) and clarity

of zones, they were categorized into strongly (+++), moderate (++) and weakly (+) haemolysin producing strains.

This study identified all 17 isolates of *L. monocytogenes* to be positive for haemolysin production. Of these haemolytic isolates six isolates from pig (4 pork and 2 faecal) showed strong haemolytic activity, other six isolates of faecal origin (2 goat and 4 porcine) revealed a zone of moderate haemolytic activity whereas, remaining five goat isolates (3 chevon and 2 faeces) showed weak hemolytic zone (Fig. 3). Thus they were designated as pathogenic.

Christie Atkins Munch Petersen (CAMP) test

All the isolates showed positive reaction with *S. aureus* which appeared as zone of enhanced haemolysis extending about 2 mm from the test isolates and within the strongly haemolytic zone due to growth of the *S. aureus* (MTCC 3160) culture and negativity towards *R. equi* (MTCC 1135) (Fig. 4). The isolates showing strong haemolysis towards *S. aureus* were interpreted as CAMP positive pathogenic *L. monocytogenes*.

Phosphatidyl- inositol specific phospholipase-C (PI- PLC) activity assay

This study designated all the *L. monocytogenes* isolates to be positive for PI-PLC activity by demonstrating light-blue colonies on *L. mono* differential agar (Fig. 5).

Assessment of pathogenicity by hly A gene

All the seventeen *Listeria monocytogenes* isolates from this study successfully amplified an amplicon of 456bp targeting hly A gene thus further ascertaining its virulent characteristic (Fig. 6).

Table.1 Details of samples collection

Species	Type of sample	No. of sample	Place	Total
Goat	Meat	50	Bhandewadi slaughter house Nagpur	200
	Faeces	50		
	Blood	50		
	Milk	50	Farm at NVC and Borgaon (Nagpur)	
Pig	Meat	50	Immamwada slaughter house Nagpur	150
	Faeces	50		
	Blood	50		
			Total	350

Table.2 Antibiotics disc concentration in µg (mcg) per disc

Sr. No.	Antibiotics	Abbreviations	Concentrations (mcg/ unit)
1	Ampicillin	A	10
2	Cephotaxime	Ce	30
3	Ceftriaxone	CTR	5
4	Cloxacillin	Cx	30
5	Erythromycin	E	15
6	Gentamicin	G	5
7	Kanamycin	K	30
8	Norfloxacin	Nx	10
9	Oxytetracycline	O	30
10	Penicillin-G	P	10
11	Rifampicin	R	5
12	Vancomycin	VA	30

Table.3 Prevalence and pathogenicity of *Listeria monocytogenes* in goat and pig samples

Species	Type of sample (50 each)	No. of <i>Listeria</i> positive samples	Overall prevalence (%)	Pathogenicity			
				Haemolysis on SBA	CA MP	PI- PLC	<i>hlyA</i> PCR
Goat (50)	Chevon	3	14% (7/50)	+	+	+	+
	Milk	0					
	Blood	0					
	Faeces	4		+	+	+	+
Pigs (50)	Pork	4	20% (10/50)	+	+	+	+
	Blood	0					
	Faeces	6		++	+	-	+

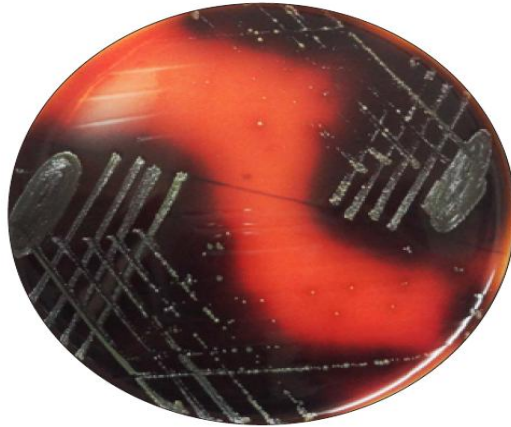


Figure.1 Characteristic colonies of *Listeria* on PALCAM

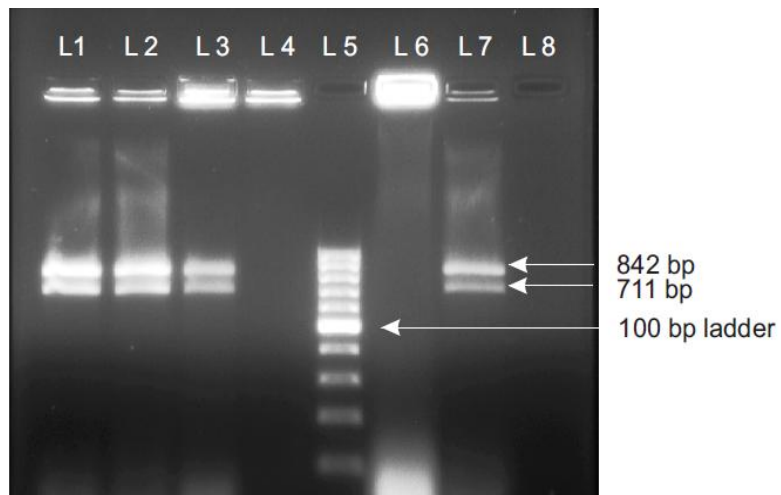


Figure.2 Multiplex PCR of *Listeria* isolates



Figure.3 Haemolysin production on sheep blood agar

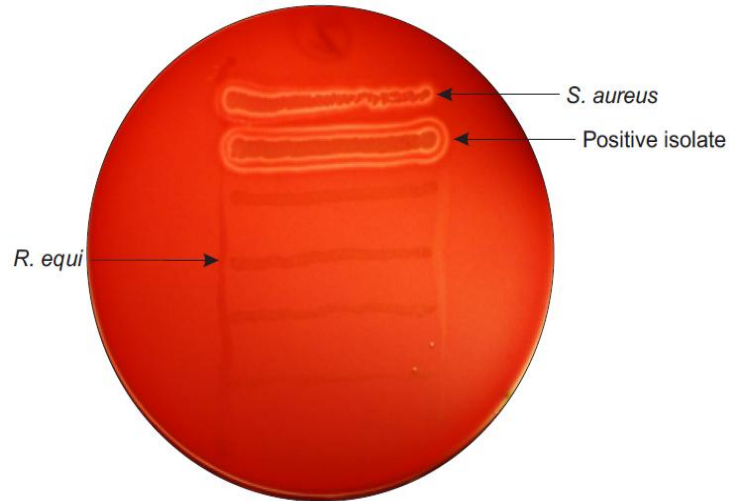


Figure.4 Christie, Atkin Munch Petersen (CAMP) test

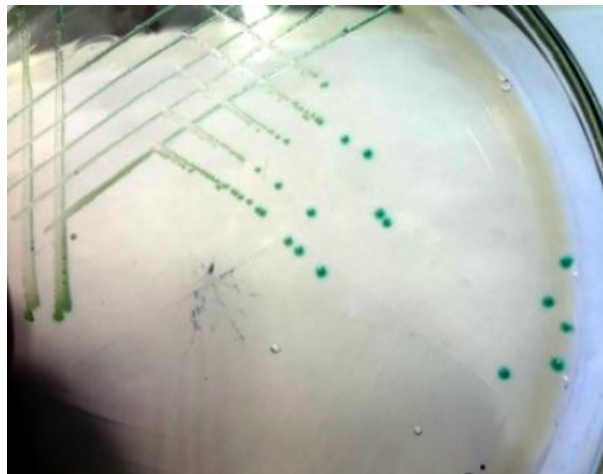


Figure.5 PI-PLC production by *Listeria monocytogenes* isolates

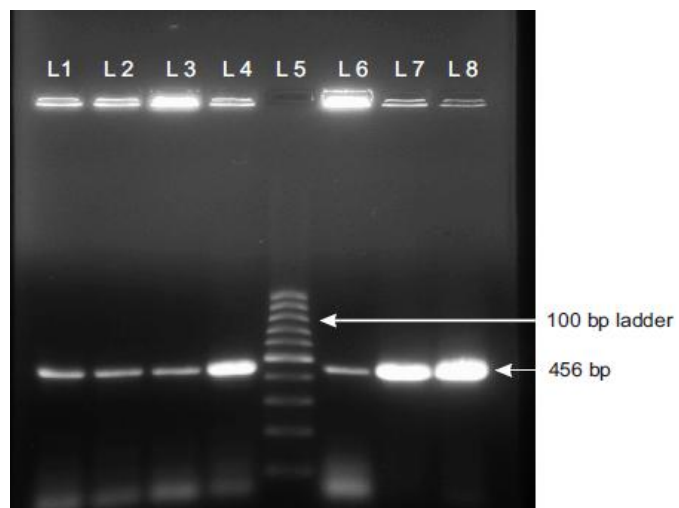


Figure.6 PCR for *hly* gene

Antibiotic sensitivity test

Antibiogram study of all (17) *Listeria monocytogenes* isolates exhibited inhibitory zones with variable activity. Of all *L. monocytogenes* isolates 52.94% were resistant to Kanamycin, followed by 47.05% for Rifampicin and 35.3% towards Cephotoxim. Maximum sensitivity was observed for Penicillin-G and Cloxacilin (76.47% each) followed by Oxytetracycline and Ceftriaxon (52.94 %), Ampicillin (41.17 %), Erythromycin (17.65 %) and 5.9 % towards Norfloxacin and Gentamicin. 100% moderate degree of sensitivity was observed towards Vancomycin followed by 94.11% each towards Norfloxacin and Gentamicin, while 82.35 % for Erythromycin.

The overall observations on prevalence among slaughtered goats and pigs in Nagpur region could be discussed with the findings of earlier works conducted among food animals slaughtered in the same region. Bharate *et al.*, (2011), Shelke *et al.*, (2011) and Suryawanshi *et al.*, (2011) in their study reported 2%, 5 % and 1.98 % positivity respectively in goats whereas, Dudhe *et al.*, (2012) recorded 8% and 12 % prevalence of *Listeria monocytogenes* among goats and pigs respectively from this region.

These workers also reported variability in xylose fermentation among the isolates of *L. monocytogenes* as observed in the present study. The findings on the prevalence of pathogenic *Listeria monocytogenes* in chevon are also supported with the recorded 6.66% and 7.08 % positivity for *Listeria monocytogenes* in chevon sampled from Bareilly (UP), India (Barbuddhe *et al.*, 2000; Bhanu Rekha *et al.*, 2006) respectively. However, the obtained positivity in chevon and pork in this study was on higher side as compared to earlier works conducted in the region by Katre *et al.*, (2009); Zade and Karpe

(2010) who recorded absence of *L. monocytogenes* among chevon and pork samples. The variation might have been resulted due to variations in isolation technique as they employed combination of UVM and DRIA while the present study used UVM and PALCAM. The reported prevalence in chevon in present study was higher as compared to Suryawanshi *et al.*, (2011) who reported 1.98 % among chevon samples employing UVM and PALCAM could be due to variation in sources, places and as such occurrence of pathogen during the period of study.

The present study reported no cultural and molecular prevalence of *Listeria monocytogenes* in blood. This observation is in absolute agreement with the reports of 0% prevalence reported in goats of Gujarat (Brahmbhatt and Anjaria, 1993) and Bareilly, UP (Elezebeth *et al.*, 2007). Previous studies from Nagpur region also stated zero isolation from goat blood samples (Suryawanshi *et al.*, 2011; Dudhe *et al.*, 2012) that could be attributed to the fact that septicaemia is relatively uncommon in listeriosis (Low and Donachie, 1997). However, in Uttar Pradesh 1.77% positivity of *L. monocytogenes* in goat blood samples has also been documented (Bhanu Rekha *et al.*, 2006). Similarly, absence of *Listeria* species in goat milk could be compared with the findings of Suguna *et al.*, (2012) who found no *Listeria* species in goat milk samples from farm at Malaysia. However, some previous studies from the region reported 5.1 % prevalence of *L. monocytogenes* in raw milk samples (Kalorey *et al.*, 2008). This variation might be attributed to the large sample size of 2060 milk samples. Also, the source of *Listeria* in milk is mainly attributed to the extraneous sources of contamination (Kalorey *et al.*, 2008) while Dudhe *et al.*, (2012) recorded 4% positivity in pig blood samples from the region.

The inconsistency of hemolysin production by *L. monocytogenes* isolates in the present study is in agreement with the finding of Tabouret *et al.*, (1991) and Nunes and Hofer (1994). The variation in haemolytic activity of *L. monocytogenes* isolates can be corroborated with the findings of Bharate (2011), Shelke (2011), Suryavanshi (2011) and Dudhe (2012) who reported the strong, moderate and weak haemolytic activity amongst the isolates of *L. monocytogenes* from buffaloes, cattle, goats and pigs slaughtered in the same region. This confirms the observation regarding perpetuation of variable haemolytic strains of *L. monocytogenes* in the same region. The variations among the production of haemolysin can be attributed to the variations at the genotypic level among strains itself as the hly A encode the production of haemolysin in case of *L. monocytogenes* (Gouws and Liedemann, 2005 and Jallewar *et al.*, 2007). The observation of 100% haemolytic *L. monocytogenes* isolates noted in present study is in agreement with Golden *et al.*, 1988), Barbuddhe *et al.*, (2000), Lacier and Centorbi (2002), Molla *et al.*, (2004), Chaudhari *et al.*, (2004), Becker *et al.*, (2006), Bhanu Rekha *et al.*, (2006), Jallewar *et al.*, (2007) and Elezabeth *et al.*, (2007) who stated 100 % hemolytic activity by the field strains of *L. monocytogenes* from various parts of India. Overall inconsistency in hemolysin production may be found because of composition of medium, or a loss of ability to generate hemolysin. Further, the variety of toxins could also contribute some of the contradictory results.

The potentials of CAMP test as in-vitro pathogenicity assay has been exploited by many workers including Barbuddhe *et al.*, (2000), Molla *et al.*, (2004), Becker *et al.*, (2006), Bhanu Rekha *et al.*, (2006), Nayak *et al.*, (2010) and Gebretsadik *et al.*, (2011). The findings of all these workers are in

accordance with the results obtained in our study. Prevalence of CAMP positive *L. monocytogenes* in the present investigation in this region correlates with earlier studies by Chaudhari *et al.*, (2004), Kalorey *et al.*, (2006), Jallewar *et al.*, (2007), Bharate (2011), Suryawanshi (2011), Mathakiya *et al.*, (2011), Shelke *et al.*, (2011) and Dudhe *et al.*, (2012), who isolated *L. monocytogenes* strains from buffaloes, goats, cattle and pigs respectively showing CAMP positivity.

Cent per cent PI-PLC activity as observed in the present investigation is in agreement with the findings of Sonegaonkar (2009) and Yadav *et al.*, (2010) who also reported 100 % *L. monocytogenes* positive for PI-PLC from milk, milk products and human isolates respectively. When compared with the earlier works conducted in the region, 100% PI-PLC ability was observed among all *L. monocytogenes* isolates (Suryawanshi 2011; Shelke *et al.*, 2011; Dudhe *et al.*, 2012). However, variations were also noted by many workers namely, Shakuntala *et al.*, (2006), Kalorey *et al.*, (2008) and Kaur *et al.*, (2010) who observed 16.66%, 4.36 % and 29.03 % *L. monocytogenes* positivity for PI-PLC from organized farm at Uttar Pradesh, bovine raw milk samples from Vidharbha region, Maharashtra and human samples respectively. Previous work from the same region also reported PI-PLC negativity of *L. monocytogenes* isolates (Bharate, 2011). This may be due to absence of plc-A gene or no co-ordination of genes encoding haemolysis and PI-PLC activity or it may be because of lack of virulence determinant due to mutation in strain of *L. monocytogenes* (Notermans *et al.*, 1991, Shakuntala *et al.*, 2006 and Kaur *et al.*, 2010).

The observations on the prevalence of kanamycin, rifampicin, cephotaxime and penicillin resistant *L. monocytogenes* in the present study are supported by the previous

studies by Jaulkar (2009), Zade and Karpe (2010), Bharate (2011), and Suryawanshi *et al.*, (2011) who also recorded resistance of *Listeria monocytogenes* towards aforementioned antibiotics from the same region. However, the degree of resistance varies among all these studies considerably. In general observations and deviations in the antibiotic resistance as well as sensitivity pattern with that of earlier works have been recorded in the present investigation. This can be probably due to the frequent use of these antibiotics for therapeutic use among food animals under same geographical area which might have contributed to the increased resistance of *Listeria* species against these commonly used drugs. These deviations in the antibiotic resistance pattern can also be attributed to the fact that antibiotic resistance is encoded by the plasmid which has capability to transfer from one strain to another (Mayer, 1988).

The presence of virulent and multiple drug resistant *L. monocytogenes* in goat and pig slaughtered for human consumptions highlights an important risk of foodborne listeriosis and public health concern for chevon and pork consumers of this region.

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Conflict of interest

The authors declare that they have no competing interests.

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