



Determination of Prevalence and In-vitro Pathogenicity of *Listeria monocytogenes* in Sheep and Goats Reared by Nomads of Jammu Region of J&K

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ABSTRACT

The study was carried out to determine the prevalence and the in-vitro pathogenicity of *Listeria monocytogenes* in sheep and goats reared by nomads of Jammu region of J&K, in addition to biochemical characterization of isolates. A total of 125 samples comprising of sheep and goat faecal samples and their rectal swabs were collected. Isolation of *Listeria* species involved the enrichment of the samples followed by plating on selective culture media and identification of the organism. Out of 125 samples collected, 32 (25.6%) samples were found positive for *Listeria* species. Out of these 13 (10.4%) were identified as *L. monocytogenes* and 19 (15.2%) were the other *Listeria* species. All the biochemically confirmed *Listeria monocytogenes* (n=13) isolates were hemolytic and CAMP positive but out of 13 *Listeria monocytogenes* isolates only 10 isolates were positive for PI-PLC assay. Antibiotic sensitivity test revealed sensitivity towards Ampicillin, Ciprofloxacin, Livofloxacin (100%), Enrofloxacin (76.92%) and Erythromycin (69.23). From this study we can conclude that sheep and goats of nomads harbour the *Listeria* species in their gastrointestinal tract. Under such conditions there is every apprehension that the handlers associated with such animals can contract listeriosis, which in turn may lead to an outbreak. So, the need of an hour is to impart the education of proper hygienic practices in public in general and the animal handlers in particular.

Keywords: *Listeria monocytogenes*, Prevalence, biochemical, in-vitro pathogenicity, antibiotics, hemolytic, sheep, goats

Listeria is a Gram-positive bacterium first described in 1926 in Cambridge, United Kingdom, as a cause of infection with monocytosis in laboratory rodents and was named as *Listeria monocytogenes* in the honour of surgeon Lord Lister. *L. monocytogenes* is a facultative food borne pathogen of humans and animals (Abhay *et al.*, 2014). *Listeria* genus has fifteen species viz., *L. monocytogenes*, *L. ivanovii*, *L. fleischmannii*, *L. grayi*, *L. innocua*, *L. marthii*, *L. rocourtae*, *L. seeligeri*, *L. weihenstephanensis*, *L. welshimeri*, *L. floridensis*, *L. aquatic*, *L. cornellensis*, *L. riparia* and *L. grandensis* (Meloni, 2014) Among these *L. monocytogenes* is an emerging food borne zoonotic pathogen of high public health significance infecting animals and humans (Guillet *et al.*, 2010). *L. monocytogenes* can grow at pH ranges between 4.5 and 9.0 (optimum pH between 6 and 8) and is able to multiply in food matrices at water activity (aw) values of 0.92 and in NaCl concentrations of 12%, generally lethal to

other microorganisms. *L. monocytogenes* is a ubiquitous organism, widely distributed in the environment: the principal reservoirs are soil, forage and water (Todd *et al.*, 2011) other reservoirs include healthy humans and animals or infected domestic and wild animals (Abhay *et al.*, 2015). *L. monocytogenes* is a psychrotrophic bacterium, can multiply at low temperatures, both under aerobic and anaerobic conditions, adapt to disinfectants and adhere to various surfaces (Arevalos-Sánchez *et al.*, 2012).

L. monocytogenes is usually inhabitant of intestinal tract of mammals, birds and soil which act as source of infection via the food chain or direct contact with animals or contaminated material from diseased or healthy animals. The ruminants are the major reservoir of *L. monocytogenes* in which infection is generally asymptomatic but could be manifested as encephalitis, abortion, septicemia and mastitis with considerable economic losses. In India, *L.*



monocytogenes has been reported from a wide range of foods such as meat, milk and seafood (Barbuddhe *et al.*, 2012). Few studies reported higher occurrence of *Listeria* in slaughterhouses as well (Meloni *et al.*, 2013). Higher excretion of *L. monocytogenes* in animal faeces results in more prevalence of the organism in foods of animal origin viz. milk, milk products, meat and fish (Skovgaard *et al.*, 1988). The food products viz. vegetables (cabbage, lettuce, celery, cauliflower, broccoli), meat (poultry, chevon, mutton), meat products, fish and ready to eat food items such as milk, ice-cream, cheeses, sandwiches, bakery products have been incriminated for listeriosis in number of cases (Thunberg *et al.*, 2002; Eruteya *et al.*, 2014).

There are several factors which govern the infectivity of *Listeria* viz., internalin, listeriolysin-O, phospholipases and Act-A. The presence of these factors in *Listeria* makes it a pathogenic strain while their absence makes it non-pathogenic (Rawool *et al.*, 2007). The *Listeria* isolates were confirmed based on biochemical tests, haemolysis on blood agar, CAMP test, PI-PLC assay followed by *in vivo* pathogenicity tests and multiplex PCR to detect virulence associated genes (*plcA*, *prfA*, *hlyA*, *actA* and *iap*). The pathogenic potential of *Listeria* can be assessed by *in vivo* methods namely, chick embryo and mouse inoculation tests and *in vitro* pathogenicity tests like hemolytic activity, phosphatidylinositol specific phospholipase C (PIPLC) assay phosphatidylcholine specific phospholipase C (PCPLC) assay.

Food-borne transmission is the most important route in 85 % to 95% of human beings though direct transmission from infected animals to farm workers and veterinarians is also documented (McLauchlin *et al.*, 1994). In India, beside the reports of prevalence of *Listeria* species among a variety of animals and foods of animal origin (Katre *et al.*, 2009; Sambyal *et al.*, 2016) few cases of human listeriosis have also been recorded (Malik *et al.*, 2007; Peer *et al.*, 2010). *L. monocytogenes* has been isolated by different workers (Willayat *et al.*, 2005) from cases of abortions and encephalitis in sheep and goats in different parts of the country.

In human beings, especially the pregnant women, the unborn child, elderly persons, alcoholics, drug abusers, diabetics, patients receiving treatments which alter their natural immunity, AIDS patients, and patients with malignancy are at greater risk of acquiring *Listeria*

infection (Willayat *et al.*, 2005; Drevets *et al.*, 2008). Moreover, *L. monocytogenes* has the potential to cause many other life threatening disease and most of the cases of human listeriosis are associated with contaminated foods (Andritsos *et al.*, 2013). The *L. monocytogenes* is a well established cause of mastitis, abortion, encephalitis, infertility, repeat breeding, and septicemia in cattle (Deb *et al.*, 2013). However, *Listeria* infection can also occur in healthy individuals (Valk *et al.*, 2005). The disease in humans is characterized mainly by meningitis, meningoencephalitis, rhombencephalitis, abortion and septicaemia (Antal *et al.*, 2005).

The major reservoirs of *Listeria* spp. are animals and environment. Several studies have been conducted in India in order to evaluate the presence of *Listeria* species in foods and varying prevalence results have been obtained from different regions of India. In Jammu region of Jammu and Kashmir State there is scanty of data with very few studies (Sambyal *et al.*, 2016) and more prevalence studies are required for generating data from small ruminants particularly in the regions where the handlers live with their livestock, in some cases even under the same roof. The objective of the study is to determine the prevalence of *Listeria monocytogenes* in sheep and goat flocks of nomads and *in vitro* studies on pathogenicity potential of *Listeria monocytogenes* isolates.

MATERIALS AND METHODS

Bacteria

The standard strains of *Listeria monocytogenes* 4b (pathogenic) (MTCC 1143), *Staphylococcus aureus* (MTCC 1144) and *Rhodococcus equi* (MTCC 1135) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India for standardization of techniques. All the strains were maintained by periodic subculturing in brain heart infusion (BHI) agar as per the instructions of IMTECH.

Sample collection

A total of 125 samples (Table 1) comprising of Sheep faeces (n=33), Sheep rectal swabs (n=30), Goat faeces (n=32), Goat rectal swabs (n=30) were collected.

Table 1: Collection of samples

Sl. No.	Type of Samples	No. of Samples	Source
1	Sheep		
	Fecal samples	33	Jammu
	Rectal swabs	30	Jammu
2	Goat		
	Fecal samples	32	Jammu
	Rectal swabs	30	Jammu
	Grand Total	125	

Samples were collected from the flocks of sheep and goat of nomadic community partially settled in Jammu and surrounding areas, as per the procedure recommended by International Commission on Microbiological Specifications for Foods. The samples were collected aseptically and transported to the laboratory under chilled conditions and were stored at 4°C till analysed.

Isolation of *Listeria* species

The isolation was carried out as per USDA protocol (USDA, 2013). It involved the enrichment of the samples followed by plating on selective culture media and identification of the organism.

Two stage enrichment procedure comprised of primary enrichment followed by secondary enrichment described by (McClain *et al.*, 1988). 1 gm of faeces were inoculated with 9 ml of University of Vermont medium-I (UVM-I) (Hi-media, Mumbai) and were incubated at 30°C for 24-36 hours for primary enrichment. Enriched inoculum (0.1 ml) from UVM-I was then transferred to 10 ml of University of Vermont medium-II (Hi-media, Mumbai) and incubated at 30°C for 48 hours for secondary enrichment. Inoculum (0.1 ml) from UVM-II was streaked directly on *Listeria* Oxford medium base, modified (Hi-media, Mumbai) and incubation at 30°C for 48 hours. Presumptive *Listeria* colonies of typical small, round greyish blackish colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis (Fig. 1) were picked up for identification (2-5 from each plate). These colonies were further purified on brain heart infusion (BHI) agar (Hi-media, Mumbai).

Biochemical characterization and identification of isolates

A single isolated colony from *Listeria* Oxford medium

base, modified was inoculated in fresh BHI broth (Hi-media, Mumbai) and incubated at 37°C for 18 h. The freshly grown culture was then studied for their morphological and biochemical characters. Morphology was observed under light microscope while, *Listeria* specific biochemical tests such as catalase, oxidase, characteristics tumbling motility at 20–25°C and fermentation of sugars (mannitol, rhamnose and α -methyl D – mannoside) were performed. *In vitro* pathogenicity test was carried out by haemolysis on 5% sheep blood agar (SBA) (Hi-media, Mumbai), Christie, Atkins, Munch–Petersen (CAMP) test with *Staphylococcus aureus* (MTCC 1144) and *Rhodococcus equi* (MTCC 1135), and phosphatidylinositol phospholipase C activity (PI-PLC).



Fig. 1: *Listeria* colonies on *Listeria* Oxford Medium Base, Modified

In vitro pathogenicity analysis of *Listeria* isolates

Haemolysis on sheep blood agar (SBA)

All the *Listeria* isolates were tested for the type and the degree of haemolysis on SBA. The isolates were streaked onto SBA plates and incubated at 37°C in a humidified chamber for 24 hrs and examined for haemolytic zones around the colonies. The characteristic narrow zone of β -haemolysis was the characteristic of *L. monocytogenes*.

Christie, Atkins, Munch-Peterson (CAMP) test

All the haemolytic *Listeria* isolates were tested by CAMP test as per the method of BIS (1994) with some modifications. Briefly, the standard strain of *Staphylococcus aureus* (*S. aureus*) (MTCC 1144) and *Rhodococcus equi* (*R. equi*) (MTCC 1135) were grown overnight on sheep blood agar (SBA) plates at 37°C and one colonies of each were again streaked onto freshly prepared SBA plates having 5 percent horse blood in a manner that these were wide apart and parallel to each other. Subsequently, the *Listeria* isolates were streaked onto these plates at 90° angle and 3 mm apart from *S. aureus* and *R. equi* strains and incubated at 37°C for 24 hrs. The *Listeria* isolates forming enhancement of haemolytic zone with *S. aureus* on SBA were characterized as *L. monocytogenes* and others forming enhanced haemolytic zone near the *R. equi* streak were suspected to be *L. ivanovii*.

Phosphatidylinositol-specific phospholipase C (PI-PLC assay)

All the confirmed *Listeria monocytogenes* isolates were assayed for PI-PLC activity as per the method of Notermans (Todd et al., 1994). The *Listeria monocytogenes* isolates were grown overnight onto L. mono Confirmatory Agar Base (Hi-media, Mumbai) with *Listeria* mono Selective Supplement I & II and *Listeria* mono Enrichment Supplement II at 35-37°C. Phospholipase C enzyme produced by virulent *L. monocytogenes* and *L. ivanovii* hydrolyses the phosphatidylinositol substrate added to the medium and results in the formation of an opaque halo around the colonies. The supplements also contains α -Methyl D-mannoside, whose fermentation by *L. monocytogenes* produced yellow coloured colonies while *L. ivanovii* cannot ferment this sugar so the colonies are purple coloured. Thus, differentiation between *L. monocytogenes* and *L. ivanovii* can be achieved on the basis of α -Methyl D-mannoside utilization. So the PI-PLC positive *L. monocytogenes* formed yellow coloured colonies with halo and *L. ivanovii* formed purple coloured colonies with halo.

Antibiogram of *Listeria monocytogenes* isolate

All the confirmed *Listeria monocytogenes* isolates were subjected to antibiotic sensitivity by disc diffusion method

and antibiotic disc used were Penicillin-G, Ampicillin, Amikacin, Ceftriaxone, Cephalexin, Ciprofloxacin, Enrofloxacin, Erythromycin and Livofloxacin.

RESULTS AND DISCUSSION

Isolation and identification of *Listeria monocytogenes*

Out of 125 samples collected, 32 (25.6%) samples were found positive for *Listeria* spp. Out of these, only 13 (10.4%) were identified as *L. monocytogenes* and 19 (15.2%) were the other *Listeria* species (Table 3). All the 32 isolates were Gram-positive, Catalase positive, methyl red and voges-proskauer tests positive and out of these only 19 isolates produced acid from rhamnose and α -methyl D-mannoside, while the isolates failed to produce acid from mannitol. A typical umbrella like growth was observed along the stab in the culture tubes in 13 isolates out of biochemically confirmed 32 *Listeria* isolates, thus confirming them to be *Listeria monocytogenes*.

Table 2: Results of PI-PLC pathogenicity assay of *Listeria monocytogenes* isolates

Source of Samples	<i>Listeria monocytogenes</i>
No. of isolates tested	No. of PI-PLC positive
Sheep and Goat Faeces 5	3
Sheep and Goat Rectal swab 8	7

A typical β -haemolysis with a narrow zone was exhibited by 13 *Listeria monocytogenes* isolates, while a well-defined clear zone was present in 9 isolates. The remaining 10 isolates were non hemolytic. A total of 22 hemolytic isolates as obtained through haemolysis on SBA, were subjected to CAMP test with *Staphylococcus aureus* (*S. aureus*) (MTCC 1144) and *Rhodococcus equi* (*R. equi*) (MTCC 1135). A total of 13 isolates showed enhancement of haemolytic zone with *S. aureus* on SBA and were characterized as *Listeria monocytogenes* but there was no haemolytic zone shown between *Listeria monocytogenes* and *R. equi* (Fig. 2). Other 9 isolates showed enhancement with *R. equi* and were suspected to be *Listeria ivanovii*. The 13 *Listeria monocytogenes* isolates confirmed by motility test, haemolysis on SBA and CAMP test were assayed for PI-PLC activity. Out of 13 *Listeria monocytogenes* isolates 10 were positive for PI-PLC assay and exhibit an opaque halo around the yellow coloured colonies (Fig. 3).

Thus, it was concluded that these 10 *L. monocytogenes* isolates contain the virulent *plc* gene (Table 2).

Antibiogram pattern

Antibiogram pattern of *L. monocytogenes* isolates

All the 13 confirmed isolates of *L. monocytogenes* revealed high sensitivity towards Ampicillin, Ciprofloxacin and Livofloxacin (100% each). High sensitivity was also observed toward Enrofloxacin (76.92%) and Erythromycin (69.23%), where as moderate sensitivity was observed towards Amikacin (61.54%). The isolates show resistance towards Penicillin-G (100%), Cephotaxime (69.23%) and Ceftriaxone (53.85%) (Table 4).

Table 3: Prevalence of *Listeria monocytogenes* in various types of samples collected from sheep, goat and nomadic handlers

Sl. No.	Types of Samples	No. of Samples	Total <i>Listeria</i> spp. isolates	No. of <i>L. monocytogenes</i> Isolates	(%)
1	Sheep faeces	33	7	2	6.06
2	Sheep Rectal swabs	30	8	3	10.00
3	Goat faeces	32	9	3	9.37
4	Goat Rectal swabs	30	8	5	16.66
	Total	125	32	13	10.4

Prevalence of *Listeria monocytogenes* in sheep and goat flocks of nomads has not been studied. The majority of nomads prefer to consume traditionally produced foods from the milk of goats. Furthermore, most goat and sheep farms in J & K state do not take adequate hygiene precautions and animals live in a natural environment together with people. Therefore, we aimed to determine the prevalence of *Listeria monocytogenes* in sheep and goats flocks belonging to nomadic community. The overall prevalence of *L. monocytogenes* in the present study was 10.4% which is higher than some of the studies conducted at the International and National levels (Kalender *et al.*, 2003; Hasöksüz *et al.*, 2000; Yadav *et al.*, 2009).

Study conducted by Yadav and Roy in 2009 in Gujarat revealed a prevalence of 0.7 per cent of *L. monocytogenes* in small ruminant faeces (Yadav *et al.*, 2009). In another study, it was found that 2.94 percent samples of sheep faeces were positive for *Listeria* species out of which *L. monocytogenes* was present in 0.58 percent samples (Kalender *et al.*, 2003). *L. monocytogenes* has also been isolated in 1.11 percent sheep faecal samples in Turkey (Hasöksüz *et al.*, 2000).

Table 4: Antibiogram pattern of pathogenic *Listeria monocytogenes* isolates

Sl. No.	Antimicrobial agents (Qty.)	Antibiotic Sensitivity Pattern		
		Sensitive	Intermediate	Resistance
2	Ampicillin (10mcg)	13 (100.00%)	0 (0.00%)	0 (0.00%)
3	Amikacin (30mcg)	8 (61.54%)	5 (38.46%)	0 (0.00%)
4	Ceftriaxone (30mcg)	0 (0.00%)	6 (46.15%)	7 (53.85%)
5	Cephotaxime (30mcg)	0 (0.00%)	4 (30.77%)	9 (69.23%)
6	Ciprofloxacin (5mcg)	13 (100.00%)	0 (0.00%)	0 (0.00%)
7	Enrofloxacin (10mcg)	10 (76.92%)	3 (23.08%)	0 (0.00%)
8	Erythromycin (15mcg)	9 (69.23%)	4 (30.77%)	0 (0.00%)
9	Livofloxacin (5mcg)	13 (100.00%)	0 (0.00%)	0 (0.00%)

In Jammu, sheep and goats are mostly reared by nomads and small marginal farmers under unhygienic conditions thus creating condition conducive for its transmission especially when there is high prevalence of *L. monocytogenes*, which may be the cause of the higher prevalence in the current study in addition to low ambient temperature during the period of sampling and processing. The animals during these months are kept in closed sheds due to cold climate, thus increasing the possibility for exposure to *L. monocytogenes* via feed and fodder as reported by other workers. Besides, *L. monocytogenes* has been isolated from a variety of sources including water, sludge, soil, plants, vegetation, food, food processing plants and infected humans and animals. This may also be the reason of the high prevalence of *L. monocytogenes* in

our study, since all these factors are present in the vicinity of nomadic dwellings (Dhama *et al.*, 2013).



Fig. 2: CAMP Test for *Listeria*



Fig. 3: *Listeria monocytogenes* colonies on *Listeria* mono Confirmatory agar

The virulent character of *Listeria monocytogenes* isolates were detected by hemolysis, CAMP test and PI-PLC assay. Out of 32 *Listeria* isolates, 22 isolates were found hemolytic and CAMP positive. Out of 22 hemolytic isolates, 10 were positive for PI-PLC assay (i.e. *plc*

gene). PI-PLC assay could be a rapid and reliable *in vitro* alternative to *in vivo* pathogenicity tests. In our study it was found that from 13 *L. monocytogenes* isolates, 10 fermented α -Methyl D-mannoside and produced yellow coloured colonies with halo, which confirmed these as *L. monocytogenes* (gene *plc* positive). In a study by Rawool out of five hemolytic isolates exhibiting positive CAMP test, turned negative in PI-PLC assay (Rawool *et al.*, 2007). Sambyal in 2016 also found 30 PI-PLC positive isolates out of 75 hemolytic *Listeria* isolates and out of these 30 PI-PLC positive *Listeria* isolates 19 were *L. monocytogenes* (yellow coloured colonies with halo) (Sambya *et al.*, 2016).

All the 13 isolates of *L. monocytogenes* revealed high sensitivity towards Ampicillin, Ciprofloxacin and Livofloxacin. High sensitivity was also observed toward Enrofloxacin and Erythromycin where as moderate sensitivity was observed towards Amikacin. The isolates showed resistance towards Penicillin-G, Cephotaxime and Ceftriaxone.

Sarangi had reported similar antibiogram results, *L. monocytogenes* being highly sensitive towards Ciprofloxacin and Livofloxacin (100%). High sensitivity was also observed towards Amoxicilin and Enrofloxacin respectively, where as moderate sensitivity was observed towards Chloramphenicol and Amikacin. The isolates show resistance towards Oxytetracyclin, Gentamycin, Penicillin G, Tobramycin, Cephotaxim, Cephalexin and Ceftriaxone (Sarangi *et al.*, 2012).

In another study conducted by Khan, *L. monocytogenes* showed (33.3%) resistance to penicillin (20.8%) resistance to chloramphenicol and tetracycline. To the ampicillin, ciprofloxacin, cephalothin, gentamycin and trimethoprim a lower range of resistance from 4.1-12.5% was observed. In this study, ciprofloxacin and gentamycin were observed as most effective antibiotic against tested isolates to which only 4.1% and 8.3% resistance were observed respectively. The results of this study are very close to our findings (Khan *et al.*, 2014).

The antibiotic resistance of the pathogen is a serious public health concern. Recent reports indicate the evolution of *L. monocytogenes* towards the antibiotic resistance (Soni *et al.*, 2013). The results of antimicrobial susceptibility testing in the present study indicate that there is a high resistance of *Listeria monocytogenes* against Pencillin-G (100%),

followed by Cephalexin (69.23%) and Ceftriaxone (53.85%) that could be due to the acquisition of mobile genetic elements such as self-transferable and mobilizable plasmids and conjugative transposons and may be due to under dose self medication of nomads which has been observed during the course of study.

Since listeriosis is transmitted primarily via foods of animal origin, excretion of animals and from their environment. So the presence of *Listeria monocytogenes* in faeces of reservoir animals (particularly small ruminants) and raw food products has an important public health implication especially in developing countries, where there is widespread and uncontrolled use of antibiotics.

From this study we can conclude that sheep and goats of nomads harbour the *Listeria* species in their gastrointestinal tract. Under stressful conditions may cause listeriosis both in animals and human beings. So the knowledge of proper hygienic management practices and that of transmission of zoonotic diseases in the community is an important tool in socioeconomic upliftment process.

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