

Research Article

Prevalence of *Listeria* spp. in Animals and Associated Environment

Abhay Raorane¹, Swapnil Doijad¹, Shilpa Katkar², Ajay Pathak¹, Krupali Poharkar¹, Zunjar Dubal¹, Sukhadeo Barbuddhe^{1*}

¹Indian Council of Agricultural Research Complex for Goa, Old Goa, Goa 403402 India; ²Mumbai Veterinary College, Parel, Mumbai 400 012 India
*Corresponding author: barbudhesb@yahoo.com

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ABSTRACT

A total of 215 samples, comprising of both clinical (n=182) and farm environment (n=33), were screened to determine the prevalence of *Listeria* spp. in different parts of Konkan region, India. Out of these, 27 (12.55%) samples were found positive for *Listeria* spp. The isolates were further characterized using phenotypic assays (Hemolysis test, Christie Atkins Munch-Petersen test (CAMP) and growth on Agar *Listeria* acc. to Ottaviani & Agosti (ALOA)) and genotypic assays. The isolates were confirmed as *Listeria monocytogenes* 12(5.11%), *L. ivanovii* 2(0.93%), *L. innocua* 11(5.11%), *L. seeligeri* 2(0.93%) and *L. welshimeri* 1(0.46%). The *L. monocytogenes* isolates were recovered from clinical cases in sheep, goat and pig while one isolate was obtained from pig rearing environment. Serotyping of *L. monocytogenes* revealed 5 isolates to be of serotype 4b and 6 of serotype 1/2b. *L. monocytogenes* isolates were sensitive to ampicillin, doxycycline, ciprofloxacin, vancomycin and intermediate resistance towards chloramphenicol, penicillin and gentamicin. The study shows the prevalence of the *L. monocytogenes* in the clinical cases and associated farm environment of the Konkan region. However, further study is necessary to determine whether farm environment acts as potential reservoir transferring *L. monocytogenes* to animals.

Key Words: *L. monocytogenes*, Antibiotics sensitivity tests, Hemolysis, CAMP, ALOA

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INTRODUCTION

L. monocytogenes is Gram positive, facultative food borne pathogen of humans and animals (Dhama et al., 2013). The genus *Listeria* includes 10 species; *L. monocytogenes*, *L. ivanovii*, *L. grayi*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. marthii*, and *L. rocourtiae*, (Graves et al., 2010; Leclercq et al., 2010) including two recently identified species, *L. fleischmannii*, and *L. weihenstephanensis* (Halter et al., 2013; Bertsch et al., 2013). Out of these, *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and animals (Guillet, 2010). The *L. monocytogenes* is widely distributed in the environment and has been isolated from a variety of sources including water, sludge, soil, plants, vegetation, food, food processing plants and infected humans and animals (Liu, 2008; Dhama et al., 2013).

The listeriosis is a rare but serious food-borne disease as it exhibits 20–30% mortality, 91% hospitalization and 50% neonatal death rates (Low and Donachie, 1997; Kathariou, 2002). In human, the early stage of infection by *L. monocytogenes* generally displays initial flu-like symptoms such as chilling, nausea, fever, gastroenteritis etc. Untreated cases may lead to septicemia, meningitis, encephalitis, abortion and occasionally death (Barbuddhe et al., 2008). *Listeria* infection in animal shows broad range of symptoms from asymptomatic infection to uncommon cutaneous lesions or various focal infections such as conjunctivitis, urethritis, endocarditis, and severe disturbance of the gait,

followed by death. The *L. monocytogenes* is a well-recognized cause of mastitis, abortion, repeat breeding, infertility, encephalitis, and septicemia in cattle (Barbuddhe et al., 2008; Deb et al. 2013).

The infection by *L. monocytogenes* is generally transmitted through contaminated food (Farber and Peterkin, 1991). Generally industrially processed foods such as raw meat, fish, milk, milk-related products has been linked for the listeriosis (Rocourt, 2000; Lyytikäinen et al., 2006; Kvistholm Jensen et al., 2010; Goulet et al., 2012; Dhama et al., 2013).

Heavy use of antibiotics as a growth promoter for farm animals and injudicious use of antibiotics accelerated evolution of bacteria towards antibiotic resistance. Since bacteria have the remarkable ability to develop resistance against antibiotics, bacterial species such as *Listeria* has also evolved towards multiple antibiotics resistance (Charpentier and Courvalin, 1999).

In India, the occurrence of listeriosis is poorly studied. Lack of awareness makes *L. monocytogenes* underdiagnosed therefore incidences in humans and animals are underestimated. Several researchers have explored incidence of listerial spp. in India from different sources (Jallewar et al., 2007; Yadav et al., 2011; Manoj et al., 1991; Dhanashree et al., 2003; Moharem et al., 2007; Kalorey et al., 2008). Also, *L. monocytogenes* cases have been reported

sporadically in humans and animals (Mokta et al., 2010; Adhikary and Joshi, 2010). The present epidemiological data is insufficient and not conclusive (Barbuddhe et al., 2012). The objective of the study is to determine the prevalence of *Listeria* spp. in Konkan region of Maharashtra, India.

MATERIALS AND METHODS

Bacteria

Standard cultures of *L. monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), and *Rhodococcus equi* (MTCC 1135) were obtained from Microbial Type Culture Collection Center, Institute of Microbial Technology (IMTECH), Chandigarh, India.

Sample Collection

A total of 215 samples were collected from Konkan region of Maharashtra, India (Table 1). Of these, 182 samples (vaginal swabs, blood, milk and faeces) were collected from domestic animals such as sheep, goat, bovines and pig, while, 33 samples were collected from cattle and pig farms surroundings (soil and floor swabs). The samples were collected in sterile containers, transported to laboratory on ice and processed for isolation of listerial spp.

Table 1: Isolation of *Listeria* spp. from clinical and environmental samples.

	No. positive <i>Listeria</i> spp	No. positive				
		Lm	Liv	Lin	Lw	Ls
Clinical						
Sheep (n=58)	10	4	0	6	0	0
Goat (n=60)	7	5	0	1	1	0
Cattle (n=30)	0	0	0	0	0	0
Pig (n=34)	5	1	2	2	0	0
Environmental						
Cattle Farm (n=18)	3	0	0	1	0	2
Pig Farm (n=15)	2	1	0	1	0	0
Total	27	11	2	11	1	2

Lm: *L. monocytogenes*; Liv: *L. ivanovii*; Lin: *L. innocua*; Lw: *L. welshimeri*; Ls: *L. seeligeri*

Isolation of *Listeria* Species

Isolation of *Listeria* spp. was attempted as per USDA-FSIS method (USDA, 2013). The samples (blood, milk, faeces and soil) were inoculated (approx. 5 ml/5 g) into 45 ml of University of Vermont Medium (UVM)-1 supplemented with acriflavin and nalidixic acid, and incubated at 37°C for 18–24 h. Vaginal and floor swabs were directly inoculated in 10 ml UVM broth. Further enrichment of the samples was carried by inoculating 0.1 ml of UVM-1 to 10 ml of UVM-2 broth. Inoculated UVM-2 broth was incubated further for 24 h at 37°C. A loopful of enriched UVM-2 broth was streaked directly on PALCAM agar for selective isolation of listerial colonies. The inoculated agar plates were incubated at 37°C for 48 h. The isolated pinpoint grayish-green colonies surrounded by black zone of esculin hydrolysis were presumed as *Listeria*. These colonies were further purified on PALCAM agar and stored in refrigerated conditions in BHI broth.

Biochemical Characterization and Identification of Isolates

A single isolated colony from PALCAM agar was inoculated in fresh BHI broth 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 (rhamnose, xylose, mannitol and α -methyl D – mannopyranoside) were performed. The isolates were compared with standard *Listeria* spp. for identification. Isolates suspected as *L. monocytogenes* from biochemical tests were further subjected to specific tests such as hemolysis on sheep blood agar (SBA), Christie, Atkins, Munch–Petersen (CAMP) test and phosphatidylinositol phospholipase C activity (PI-PLC) (Gorski et al., 2008).

Genotypic Characterization

Biochemically confirmed *L. monocytogenes* isolates were subjected to serotyping by multiplex PCR method as described by Doumith et al. (2004). The genomic DNA was extracted using DNA isolation kit (Chromos Biotech, India). Oligonucleotide primers (Doumith et al., 2004) were synthesized from Sigma. The 50 μ l PCR mixture contained 2.5 μ l of 10x PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM MgCl₂; 0.01% gelatin), 2 mM dNTP mix, 3 mM MgCl₂ and 0.3 μ M each of forward and reverse primers (*lmo0737*, *lmo2819*, *lmo2110* and *prs* gene), 2 units of Taq DNA polymerase and 50 ng of DNA template. The final volume was adjusted by sterilized deionised water. The reaction was performed in a Mastercycler epGradient (Eppendorf, Germany) with a preheated lid. PCR was performed with an initial denaturation step at 94°C for 3 min; 35 cycles of 94°C for 0.40 min, 53°C for 1.15 min, and 72°C for 1.15 min; and one final cycle of 72°C for 7 min. Five microliters of the reaction mixture was mixed with 1 μ l of gel loading buffer and separated on a 1.5% agarose gel pre-stained with ethidium bromide. The gel was visualized under Alpha-Imager gel doc system.

PCR amplification of the *hlyA* gene was performed as described by (Paziak-Domańska et al., 1999). The 50 μ l PCR mixture contained 2.5 μ l of 10x PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM MgCl₂; 0.01% gelatin), 2 mM dNTP mix, 2 mM MgCl₂ and 0.3 μ M of forward and reverse primers, 1.5 units of Taq DNA Polymerase and 50ng of DNA template. The final volume was adjusted by sterilized deionised water. The reaction mixture was subjected to an initial denaturation at 95°C for 2 min followed by 35 cycles each of 15 s denaturation at 95°C, 30 s annealing at 60°C and 1 min 30 s extensions at 72°C. It was followed by final extension of 10 min at 72°C and held at 4°C. The PCR products were tested as described above.

Antibiotic Sensitivity of Isolates

Disk diffusion susceptibility tests were performed according to the standard reference procedure of the Clinical and Laboratory Standards Institute (Anon, 2006; Altuntas et al., 2012). A single well-isolated colony of *L. monocytogenes* was transferred into 10 ml BHI broth, incubated at 37°C for 24 h, diluted 1:10 in 9 ml 0.1% peptone water to a turbidity equivalent to 0.5 McFarland standard, and spread on surface of Mueller–Hinton Agar (MHA) plate. Antibiotic discs of vancomycin (VA10), ciprofloxacin (CF5), erythromycin (E15), gentamycin (G10), penicillin–G (P10), sulphazidine (Sz 30), ampicillin (A10), oxytetracycline (O30), chloramphenicol (C30) and doxycycline (D30) were placed on the surface of each inoculated MHA plate. After incubation for 24 h at 37°C, the diameter (in mm) of the zone around each disk was measured and interpreted in

accordance with the Clinical and Laboratory Standards Institute Standards guidelines (Anon et al., 2006) to classify the antibiotic sensitivity of each isolate. *Staphylococcus aureus* ATCC 6538 was used as standard strain.

RESULTS

Isolation and Identification of *Listeria*

Out of 215 samples (clinical and farm environment) collected, 27(12.55%) samples were found positive for *Listeria* spp. After further characterization, 11 (5.11%) isolates were confirmed as *L. monocytogenes*, 2 (0.93%), *L. ivanovii*, 11 (5.11%), *L. innocua*, 1 (0.46%), *L. welshimeri* and 2 (0.93%) as *L. seeligeri* (Table 1). Except clinical samples collected from cattle, all other types of samples showed presence of *Listeria* spp. Four *L. monocytogenes* were isolated from clinical samples collected from sheep, five from goats, one each from pig and pig-associated environment. The *L. ivanovii* was isolated from a clinical case in pig. No *L. monocytogenes* or *L. ivanovii* could be isolated from cattle and associated environment.

Biochemically identified *L. monocytogenes* and *L. ivanovii* spp. were further characterized by phenotypic assays. All the *L. monocytogenes* and *L. ivanovii* exhibited weak hemolysis. In CAMP test, *L. monocytogenes* and *L. ivanovii* showed increased zone of hemolysis toward *Staphylococcus aureus* (MTCC 1144) and *Rhodococcus equi* (MTCC 1135),

respectively. All the *Listeria* spp. were tested on ALOA medium to determine the β -glucosidase and ability to produce the phospholipase enzymes. All the isolates showed blue-green color zone confirming the *Listeria* spp. while in addition, *L. monocytogenes* and *L. ivanovii* exhibited an opaque white halo of hydrolysis of phosphatidylinositol or lethicin in the medium due to phospholipases. Presence of phospholipase activity was considered as indicator the pathogenicity.

Table 2: Multiplex PCR serotyping and detection of Virulence Gene (*hly A*) of the isolates.

Isolates	Source	<i>hly A</i>	Serogroup
FC2	Fish container	+	4b, 4d, 4c
16	Blood	+	4b, 4d, 4c
22	Sheep vaginal swab	+	1/2b, 3b, 7
19	Goat vaginal swab	+	1/2b, 3b, 7
21	Sheep vaginal swab	+	1/2b, 3b, 7
26	Goat vaginal swab	+	1/2b, 3b, 7
13	Sheep vaginal swab	+	4b, 4d, 4c
14	Sheep vaginal swab	+	4b, 4d, 4c
11	Goat vaginal swab	+	4b, 4d, 4c
2	Goat vaginal swab	+	1/2b, 3b, 7
12	Goat vaginal swab	+	1/2b, 3b, 7

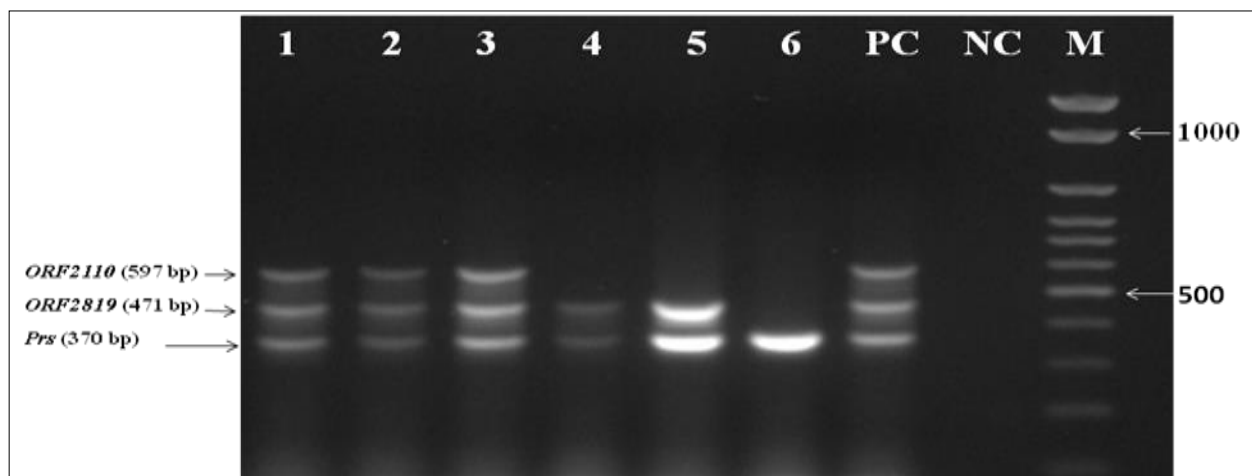


Figure 1: Multiplex PCR serotyping for determination of the serogroups of the isolates; Lane 1: Isolate FC2 *L. monocytogenes* serogroup 4b, 4d, 4c; Lane 2: 16 *L. monocytogenes* serogroup: 4b, 4d, 4c; Lane 3: 13 *L. monocytogenes* serogroup 4b, 4d, 4c; Lane 4: 22 *L. monocytogenes* serogroup 1/2b, 3b, 7; Lane 5: 19 *L. monocytogenes* serogroup 1/2b, 3b, 7; Lane 6: *Listeria* spp., Positive control (PC); *L. monocytogenes* MTCC 1143; NC: Negative control; M: 100 bp DNA ladder.

L. monocytogenes isolates were characterized for the presence of the hemolysin gene and by multiplex PCR based-serotyping. Multiplex PCR based serotyping revealed 5 isolates as serotype 4b while remaining 6 were of serotype 1/2b (Table 2, Figure 1). All the *L. monocytogenes* isolates were found to possess *hlyA* gene, which is responsible for hemolysin. In case of *L. ivanovii* genus, specific *prs* gene was amplified.

Antibiotic Sensitivity Testing

L. monocytogenes isolates were checked for their susceptibility towards the commonly prescribed antibiotics. All isolates were sensitive towards, erythromycin, oxytetracycline, ampicillin, doxycycline and ciprofloxacin and showed intermediate resistances towards the chloramphenicol,

penicillin, gentamicin and vancomycin. However, a single *L. monocytogenes* serotype 4b isolate from pig placental tissue exhibited multi-antibiotic resistance. All 10 *L. monocytogenes* isolates shown resistance to sulphazidine.

DISCUSSION

Listeria spp. are ubiquitous in nature and has been isolated from wide environmental sources (Liu, 2008). The organism possesses ability to survive in harsh conditions and therefore can persist in environment. Because of such persistence *Listeria* spp. can easily enter in food chain (Carpentier and Cerf, 2011). Of the known *Listeria* spp., *L. monocytogenes* is pathogenic to humans and animals, while *L. ivanovii* is mainly an animal pathogen. Several foodborne

outbreaks are attributed to *L. monocytogenes* (Ramaswamy et al., 2007). Therefore, food quality controlling authorities from several developed countries have enforced strict regulations over occurrence of *L. monocytogenes* in food and food products (CDC, 2012). However, such regulations are largely lacking in developing countries because of underestimated listerial scenario. In India, due to lack of awareness, burden of other traditional diseases, rare expertise, and poor reporting, the incidence of listeriosis is unknown (Dandona et al., 2004; Dandona et al., 2009). To understand the listeriosis in detail, there is a need of systematic and coordinated studies to estimate the prevalence of *L. monocytogenes* in different habitats, occurrence of listeriosis in humans as well as in animals and their concordance of occurrence and actual disease. The epidemiological studies would help in understanding of the sources of infection and persistence and their risk assessment, routes of transmission, clinical forms and allow for better management of the listerial infection.

Present study was performed to determine the prevalence of *Listeria* spp. from Konkan region of Maharashtra, India. The Konkan region contributes a great source of animal and crop originated food. *Listeria* spp. were isolated from 12.55% samples. *L. monocytogenes* and *L. ivanovii* were isolated from 5.11% and 0.93% samples, respectively. The *L. monocytogenes* isolates were mainly isolated from sheep and goats. A bacterial etiology studied in relation to abortions by over 203 sheep and goat flocks showed 5–6% of *L. monocytogenes* infections (Sharma et al., 2008). Barbuddhe et al. (2000) have reported presence of *L. monocytogenes* in 6.66% and 7.4% meat samples of goats. In India, *L. monocytogenes* has been reported from fish (Dhanashree et al., 2003; Gawade et al., 2011; Parihar et al., 2008; Das et al., 2012), meat (Doijad et al., 2010; Bramhabhatt and Anjaria, 1993), and milk (Doijad et al., 2011; D'Costa et al., 2012). In the present study, *L. monocytogenes* could not be isolated from cattle. *L. ivanovii* was isolated from pig samples. Presence of *L. monocytogenes* in animal associate–environment may lead to cause infection to animals being reared in that area, however, it warrants future investigations.

There are 12 serotypes of *L. monocytogenes* known till date, of which serotypes 1/2a, 1/2b and 4b contribute more than 98% of outbreaks (Cheng et al., 2008). Our serotyping data confirmed 6 isolated to be serotype 1/2b and 5 as serotype 4b. *L. monocytogenes* serotype 4b has been considered as cosmopolitan (Herd and Kocks, 2001; Cheng et al., 2008). Presence of such outbreak–associated serotype is of public health concern. However, more studies with large number from humans and animal clinical cases may present the actual scenario.

The antibiotic resistance of the pathogen is a significant public health concern. Recent reports suggest the evolution of *L. monocytogenes* towards antibiotic resistance (Charpentier and Courvalin 1999; Altuntas et al., 2012; Soni et al., 2013). It is suggested that the increased use of antibiotics for therapeutic purposes in animals and humans may lead to the development of antibiotic resistance (Palumbo et al., 2010; Yan et al., 2010). Depending upon different geographical area, antibiotic resistance patterns of *L. monocytogenes* in food and environmental

sources may change (Yan et al., 2010). We tested all *L. monocytogenes* isolates for their antibiotic sensitivity. A single *L. monocytogenes* serotype (4b) isolates from pig placental tissue exhibited multi–antibiotic resistance while remaining 10 isolates were sensitive towards the majority of the antibiotics studied. Interestingly, all the *L. monocytogenes* isolates were resistant to sulphadiazine. The study conducted by Dhanashree et al., (2003) found similar results wherein *L. monocytogenes* isolates which were sensitive towards commonly used antibiotics were reported.

The present study showed the prevalence of *L. monocytogenes* in clinical and environmental samples from Konkan region of Maharashtra, India. The isolates belonged to serotypes having epidemic potential. There is a possibility of transfer of this pathogen from animals to foods of animal origin and thereafter the food chain.

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