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Isolation and molecular characterization of *Listeria monocytogenes* in bovine and their environment

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Abstract
A total of 625 samples comprising of animal clinical cases (n=256), milk (n=238) and farm environment (n=131), were screened to determine the prevalence of *Listeria monocytogenes* in different parts of Nagpur region, India. The isolates were further characterized using phenotypic assays (Hemolysis test, Christie Atkins Munch-Petersen test (CAMP) and growth on Agar Listeria acc. to Ottaviani and Agosti (ALOA) and genotypic assays. Out of these, 14 (5.46%) samples from animal clinical cases, 12 (5.04%) from milk and 12 (9.16%) from environment were found positive for *Listeria monocytogenes.* Overall virulence pattern indicated presence of five genes present in three strains, *plc*A, *act*A, *hly*A, *iap* in one strain, *act*A, *hly* A, *iap*, *prf*A in twelve strains, *act* A, *hly*A, *iap* in nine strains, *hly*A, *iap*, *prf*A in five strains, *hly*A, *iap* in three strains and *hly*A in five strains. Serotyping revealed out of total 38 *L. monocytogenes* isolates tested majority of isolates (n=34) represented the group corresponding to serovars 1/2b, 3b, 4b, 4d, 4e and three isolates represented serogroup corresponding to serovars 4b, 4d and 4e while one isolate was grouped as serogroup 1/2a, 1/2c, 3a and 3c. *In vitro* antibiotic sensitivity pattern of *L. monocytogenes* indicated sensitivity to erythromycin, nitrofurantin and chloramphinicol and resistance to colistin, cloxacillin, nalidixic acid and sulfonamide. Present study showed that farm environment acts as potential reservoir for transmitting *L. monocytogenes* to animals.

Keywords: *Listeria monocytogenes*, bovine environment, virulence gene, antibiogram

Introduction
Listeria monocytogenes, a facultative intracellular pathogen, is responsible for severe foodborne infections in humans and can also cause invasive disease in many different animal species, including farm ruminants (cattle, sheep, and goats). 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, and may in this manner contaminate milk and production plants (Leite *et al.*, 2006) [59] and infect humans and animals (Dhama *et al.*, 2013) [22] Moreover, it was reported that cattle farms play a bigger role in the spread of Listeria between animals or people rather than small ruminants farms (Pritchard and Donnelly, 1999) [70] Ruminants farm animals play a key role in the persistence of *Listera spp*. in the environment via a continuous faecal –oral cycle (Vazquez- Boland *et al.*, 2001) [83] The *L. monocytogenes* is a well recognized cause of mastitis, abortion, repeat breeding, infertility, encephalitis, and septicemia in cattle (Barbuddhe *et al.*, 2008;) [8].
In India, the occurrence of listeriosis is poorly studied and threre is lack of awareness about *L. monocytogenes* under diagnosed therefore incidences in humans and animals are underestimated. Several researchers have explored incidence of *listerial* spp. in India from different sources (Dhanashree *et al.*, 2003; Moharem *et al.*, 2007; Jallewar *et al.*, 2007, Kalorey *et al.*, 2008;) [23 64, 48, 51]. Also, *L. monocytogenes* cases have been reported sporadically in humans and animals (Adhikary and Joshi, 2010) [1]. Thre is indiscriminate use of antibiotics as a growth promoter for farm animals and voracious use of antibiotics accelerated evolution of bacteria towards antibiotic resistance. *Listeria* spp. has also evolved towards multiple antibiotics resistance (Charpentier and Courvalin, 1999) [15]. Hence the aim of the study was to clarify the prevalence and source of infection of *L. monocytogenes* in the bovine environment, milk and animal clinical cases collected from bovine farms over 12 months (March 2014 to February 2015) in Nagpur region and to study the virulence pattern and antibiogram of *L. monocytogenes*.

Materials and Methods

Collection of clinical samples

A total of 625 samples were collected from bovines farms from Nagpur region of Maharashtra, India. Of these 256 samples of animal clinical cases (vaginal swabs, endocervical swabs, discharges white/ bloody and Uterine infection), 131 environmental sample (soil, feed/fodder, drinking water, tap water, drainage, manure/fertilizer) and 238 milk samples were collected from various farms of Nagpur region. The samples were collected aseptically and quickly transported to laboratory under chilled condition and stored at 4 °C until tested.

Isolation of *L. monocytogenes*

Isolation of *L. monocytogenes* was attempted as per Donnelly and Baigent, (1986) [26]

Biochemical characterization and Identification of Isolates

The Listeria isolates was carried out by morphological, biochemical and cultural characterization as per standard procedures described by Cruickshank *et al.* (1975) [19] and Cowan and steel (1993) [18] were adopted.

Molecular characterization of *L. monocytogenes*

Biochemically confirmed *L. monocytogenes* isolates were subjected to multiplex PCR targeting virulence associated genes Notermans *et al.*, (1991) [68] of *Listeria monocytogene* isolates and serotyping by multiplex PCR method as described by Doumith *et al.* (2004) [27]

Genomic DNA preparation

The *L. monocytogenes* isolates were grown in 2 ml of BHI broth overnight at 37 °C. Culture were harvested by centrifugation (8000 g for 10 min) and suspended in 400 µl of TE solution (10 mM Tris HCl ;1mM EDTA, pH 8.0) Bacteria was lysed by addition of 10 µl of proteinase K(20 mg /ml) and 100 µl of 10% SDS and followed by incubation at 37 °C for 1 hr. The cell wall debris, denatured proteins, polysaccharides and polymeric matrix were eliminated by precipitation with addition of 80 µl of 5 M NaCl and 64 µl of CTAB solution (10% CTAB in 0.7 M NaCl) and incubated at 65 °C for 10 min. DNA was purified by two extractions with Phenol: chloroform (1.1), and chloroform :isoamylalcohol (24:1). DNA was precipitated by adding 100 µl isoproponal and keep at -20 °C for 30 min and later centrifuged at 8000 g for 15 min. The pellet was washed in ethanol (70%) and air dried to remove the alcohol and resuspended in 50 µl TE buffer.

Multiplex PCR targeting virulence associated genes of *Listeria monocytogene* isolates

The reaction volume was optimized as follows:- Master mix 12.5µl, 10µM of forward and reverse primer of each set final concentration (0.1 µM each) 1 unit Taq DNA polymerase, 2.5 µl cell lysed an sterilized milliq water to make up the reaction volume. The PCR tubes containing the reaction mixture were centrifuged to get the reactants at the bottom. The reaction was performed in a Px2 (Thermo electronic corporation, USA) with a preheated lid. The cycling condition including an initial denaturation at 95 °C for 10 min. followed by 35 cycles each of 15 second denaturation at 95 °C, 30 second annealing at 60 °C and 1 min. 30 second extension at 72 °C. It was followed by final extension of 10 min. at 72 °C and 10 min. hold 4°C after the reaction PCR products were kept at -20 °C until further analysis by agarose gel electrophoresis.

About 10 ul of reaction mixture was mixed with 2 µl of gel loading buffer and separated on 1.5% agarose gel prestained with ethidium bromide. The gel was visualized under UV transilluminator (Syngene G box, UK).

Singleplex PCR targeting *prf*A gene of *Listeria monocytogenes* isolates

Singleplex PCR reaction at 25 µl reaction volume standardized as follows: 2.5 µl of 10X PCR buffer (consisting of 100 mMTris-HCl, pH 8.3; 500 mMKCl; 15 mM $MgCl_2$ and 0.01% gelatin), 2.3µl of 50 mM $MgCl_2$ (final concentration 7.5 mM), 0.5 µl of 10 mMdNTP, 20 picomole of forward and reverse primer of each set (with final concentration 0.1 picomole each), 1 unit of Taq DNA polymerase, 1.25 µl of genomic DNA and sterilized miliQ water to make up the reaction volume. The cycling condition was set in Thermal cycler (Eppendrof, Germany) as follows: Initial denaturation at 95 ºC for 10 minutes followed by 35 cycles each of denaturation at 95 ºC for 15 seconds, annealing at 60 ºC for 30 seconds, extension at 72 ºC for 1 min 30 seconds and final extension at 72 ºC for 10 minutes and hold up at 4 ºC.

Serotyping PCR

In requirement PCR reaction was set at 25 µl reaction volume and for detection of *L. monocytogenes* serotype by PCR, standardized PCR was set by using reaction mixture as follows : 2.5 µl 10X PCR buffer (Ammonium sulphate) (consisting of 100 mMTrisHCl, pH 8.3; 500 mMKCL;15 mM $MgCl_2$ and 0.01% gelatin), 0.75 µl dNTP mix (10 mM, with a final concentration of 0.2 mM), 2 µl of 25 mM $MgCl_2$ (final concentration of 0.1 µM each) and 10 µM of forward and reverse primer of each set of *Listeria* species (final concentration 0.1 µM each), 1 unit of Taq DNA polymerase, 2.5 µl of genomic DNA and sterilized miliQ water to make up the reaction volume. PCR conditions steps were set as: Initial denaturation at 94 ºC for 5 minutes followed by 35 cycles each of Denaturation at 94 ºC for 30 seconds, annealing at 54 ºC for 1 min. 15 seconds and Extension at 72 ºC for 1 min 15 seconds followed by the final extension of 10 minutes at 72 ºC. Obtained PCR reaction later on stored at -20 ºC for further analysis and also checked on agarose gel by electrophoresis as described above

Antibiotic Sensitivity of the Isolates

Disk diffusion susceptibility test was performed according to the standard reference procedure of the clinical laboratory Standards Institute (Altuntas *et al*., 2012) [3]. A single well isolated colony of *L. monocytogenes* was transferred into 10 ml BHI broth, incubated at 37 ^{0}C for 24 h, diluted 1:10 in 9 ml 0.1% peptone water to a turbidity equivalent to 0.5 Mc Farland standard, and spread on surface of Muller-Hinton Agar (MHA) plate. Antibiotic disc of chloramphenicol (C30) ciprofloxacin (Cf 5), erythromycin (E15), gentamycin (G10), penicillin–G (P10), sulphazidine (Sz30) ampicillin (A10), oxytetracycline (O30), vancomycin (VA 10) nitrofurantin (N 10), cloxacillin (Cx5), colistin (Cl 10) and nalidixic acid (N10) were placed on the surface of each inoculated MHA plat. After incubation for 24 h at 37 ^{O}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, 2006) [4] to classify the antibiotic sensitivity of each isolate.

Results

Isolation and Identification of *L. monocytogenes*:

Out of 625 samples (Bovine environment, milk and animal clinical cases), Total 38 samples were positive for *Listeria monocytogenes* from three sources, yielding an overall prevalence of 6.08%. Among these 12 (9.16%) samples were positive for *L. monocytogenes* from bovine farm environment, 12 (5.04%) from milk and 14 (5.46%) samples were found positive for *L. monocytogenes* from animal clinical cases. (Table 1)

Table 1: Isolation of *L. monocytogenes* from various sources:/ Prevalence of *L. monocytogenes* in samples of different sources

Sr. No.	Sources	Samples (n)	Number of *L. monocytogenes* Positive samples (%)	Number of other *Listeria sp.* positive samples (%)
1	Bovine environmental	131	12 (9.16%)	4 (3.05%)
a	Soil	19	04(21.05%)	1(5.26%)
b	Feed/Fodder	54	02(3.70%)	1(1.85%)
c	Water	22	01(4.45%)	--
d	Tap water	03	----	--
e	Drainage	19	02 (10.52%)	1(5.26%)
f	Silage	03	01 (33.33%)	--
g	Manure	11	02(18.18%)	1(9.09%)
2	Milk	238	12 (5.04%)	5 (2.10%)
3	Animal Clinical Cases (Vaginal swabs, endocervical swabs, discharges &white/ bloody uterine infection)	256	14 (5.46%)	3 (1.17%)
	Total	625	38 (6.08%)	12 (1.92%)

L. monocytogenes biochemically identified were further characterized by phenotypic assay. All the *L. monocytogenes* exhibited hemolysis. In CAMP test, *L. monocytogenes* showed increased zone of hemolysis towards S*taphylococcus aureus* (MTCC 1144) and *Rhodococcus equi* (MTCC 1135), respectively. On ALOA medium all the *L. monocytogenes* exhibited an opaque white halo of hydrolysis of phosphotidyliositol or lethicinin in the medium due to phospholipase. Presence of phospholipase activity was considered as indicator the pathogenicity.

L. monocytogenes isolates were further characterized for presence of Multiplex PCR (*plc* A, *act* A, *hly* A and *iap*) gene and singleplex PCR (*prf*A) gene. and multiplex PCR based serotyping. Overall PCR analysis of all 38 *L. monocytogenes* isolates revealed the variable genotypic patterns for five virulence-associated genes namely *plc* A, *act* A, *hly* A *iap*. and *prf*A Multiplex PCR study of *Listeria* monocytogenes revealed that three *L. monocytogenes* isolates were positive for all five genes *plc* A, *act* A, *hly* A, *iap* and iap genes. Amplified products of four virulence-associated genes namely, *act* A, *hly* A *iap and prf* A *and plc A, act A, hly A and iap* were detected in twelve and one isolates respecrively. eight isolates were positive for act A, *hly* A *iap* genes. Five isolates were positive for *hly* A, *iap* and *prfA* gene, three isolates were positive for *hly* A, iap and five isolates were positive for *hly* A gene. (Table 2, Fig.1)

Table 2: Frequency of virulence-associated genes in *Listeria monocytogenes* isolates recovered from different sources by multiplex PCR.

Source	No. of isolates	Amplified PCR products of virulence-associated genes detected in *Listeria monocytogenes* isolates				
		***Plc* A (1484 bp)**	***Act* A (839 bp)**	***Hly* A (456 bp)**	***Iap* (131 bp)**	***Prf* A (1040)**
Milk	2	+	+	+	+	+
	5	-	+	+	+	+
	3	-	-	+	+	+
	2	_	+	+	+	_
Animal Clinical Cases	1	+	+	+	+	+
	5	-	+	+	+	+
	1	+	+	+	+	-
	5	-	+	+	+	-
	2	-	-	+	-	-
Environment Samples	2	-	+	+	+	+
	2	-	+	+	+	-
	2	-	-	+	+	+
	3	-	-	+	+	-
	3	-	-	+	-	-

Overall PCR pattern of samples

Sr.no.	PCR Pattern	Isolates No.	Total
1	*Plc* A, *act* A, *hly A*, *iap* and *prf* A	ACC 17, M1, M2	3
2	*Act* A, *hly* A, *iap*, *prf* A	M5, M6, M7, M8, M9, E14, E15, A6, A9, A10, A12, A15	12
3	*Plc* A, *act* A, *hly* A, *iap*	A2	1
4	*Act* A, *hly* A, *iap*	E12, E13 ACC4, ACC8, ACC11 M16, M17	9
5	*Hly* A, *iap*, *prf* A	M11, M13, M14E5, E16	5
6	*Hly* A, *iap*	E3, E9, E11	3
7	*hly*A	E8a, E14, E15a ACC13, ACC14	5
	Total		38

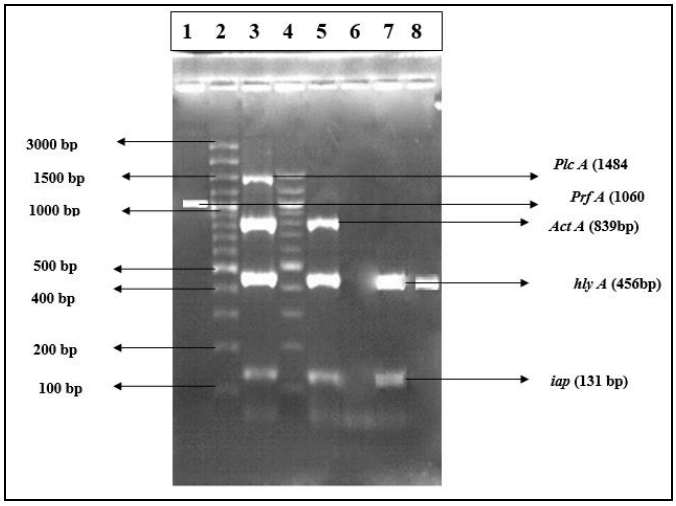

Fig 1: Agarose gel showing polymerase chain recation amplified product of 1060 bp for *prf* A gene, 1484 bp for *plc*A gene, 839 bp for *act*A gene, 456 bp for *hly*A gene and 131bp for *iap* gene in *L. monocytogenes* isolates. Lane1:isolate M-2(Singleplex *prfA* gene), Lane2:100bp plus DNA ladder, Lane3: isolate –A-2, Four-gene combination (*plcA, actA, hlyA* and *iap),* Lane 4: 100 bp DNA ladder, Lane 5: isolate E-12-three-gene combination (*actA, hlyA,* and *iap)*Lane 6: Blank; lane 7: isolate E-11 two-gene combination (*hlyA and iap*) and Lane 8:isolate A-14 *hly*A gene

Multiplex PCR based serotyping revealed Out of total 38 *L. monocytogenes* isolates tested majority of isolates (n=34) represented the group corresponding to serovars 1/2b, 3b, 4b, 4d, 4e and three isolates represented serogroup corresponding to serovars 4b, 4d and 4e while one isolate was grouped as serogroup 1/2a, 1/2c, 3a and 3c. (Table 3, Figure 2)

Table 3: Serovar grouping of the isolates.

Sr. No	Serogroup	Milk	Animal Clinical Cases	Environment	Total
1	4b. 4d, 4e	2(M8, M14)	-----	1(E3)	3
2	1/2a, 1/2c, 3a, 3c	----	------	1(E16)	1
3	1/2b, 3b, 4b, 4d, 4e	10(M1, M2, M5, M6, M7, M9, M11, M13, M16, M17	14 (ACC-2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17)	10(E5, E9, E11, E12, E13, E14, E15, E18, E24, E25)	34
	Total	12	14	12	38

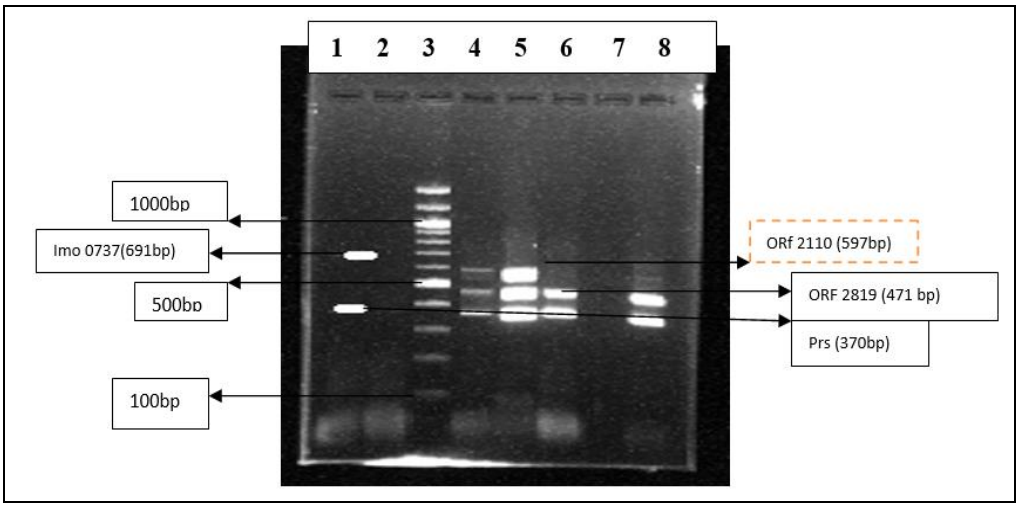

Fig 2: Agarose gel showing Multiplex PCR serotyping for determination of the serogroups of isolates obtained from bovine environment, animal clinical cases and milk. Lane1: isolates E-16 *L. monocytogenes* serogroup1/2a; Lane 2: Negative Control Lane3: 100 bp DNA ladder; Lane 4: isolates M-5 *L. monocytogenes* serogroup1/2b and 4b; Lane5: isolatesACC-*3 L. monocytogenes* serogroup 1/2band 4b; Lane6: isolate-E-*3 L. monocytogenes* serogroup 4b; Lane7: blank; Lane8: isolate M-14 *L. monocytogenes* serogroup 4b.

Antibiotic Sensitivity Testing

L. monocytogenes isolates were checked for their susceptibility towards the commonly prescribed antibiotics. All isolates were sensitive towards, chloramphinicol, erythromycin, oxytetracycline, ampicillin, and ciprofloxacin and showed intermediate resistances towards the colistin, cloxacillin, penicillin, gentamicin, nalidixic acid and vancomycin. All 38 *L. monocytogenes* isolates showed resistance to sulphazidine

Discussion

Listeriosis is one of the important bacterial diseases of animals and a zoonosis with a broad distribution; it has considerable economic and public health significance. The milk industry in India. is flourishing with cattle and buffalo playing the major role in milk production, but studies on

occurrence of the important food borne pathogens like *L. monocytogenes* in animals and its environment have not yet been carried out in detail except for a few reports (Shakuntala *et al.*, 2006, Rawool *et al*. 2007) [74, 71].

As *Listeria* spp. is highly tolerant to live under extreme pH (some cultures grow at pH
9.6), temperature (<45 °C) and salt conditions (20% (w/v) NaCl), they can be found in a variety of environments, foods and clinical samples. (Swaminathan *et al.*, 2007) [81] *L. monocytogenes* is generally considered ubiquitous, limited data on the prevalence of this pathogen in different environments have been available so far.

Present study wasundertaken to determine the prevalence of *L. monocytogenes* from Bovine environment, animal clinical cases and milk from Nagpur and in and around Nagpur region. The overall prevalence of *L. monocytogenes* from three sources were recorded as 6.08%. Among these 12 (9.16%) samples were positive for *L. monocytogenes* from Bovine farm environment, 12 (5.04%) from milk and 14 (5.46%) samples were found positive for *L. monocytogenes* from animal clinical cases respectively. High prevalence of *Listeria* spp. reported in cattle farms as high as (84.8%) to about (9.4%) in which *Listeria monocytogenes* was recorded predominantly from faeces, feed, water and environment. Beside *Listeria monocytogenes*, presence of *L. seeligeri* (from milk, water, feed and environment), *L. innocua* (from faeces, milk, feed, water and environment), *L. grayi* (from feed, faeces and environment) and *L. welshimeri* (from water and feed) has also been documented earlier (Atil *et al*., 2011) [5].

Besides milk and water, soil was also found to be the source of of *Listeria monocytogenes* contamination although it does not act as a general reservoir for *Listeria monocytogenes*. According to Weis and Seelinger (1975) [88] *Listeria monocytogenes* was prevalent at around 8.7-51.4% among surface soil samples and for 3.2-3.3%.in the soil at a depth of 10 cm. Fenlon and Shepherd (2000) [33] proposed that soil can harbour *Listeria monocytogenes* with decaying plant and faecal materials under moist conditions. Further more *Listeria monocytogenes* growth is dependent on soil type and its moisture content (Welshimer, 1960) [89] It has also reported that the agriculture soil sometimes could be contaminated with *L. monocytogenes* through sewage sludge fertilizers and manure (EPA, 2000a) [28] and (EPA, 2000b) [29]. In general, soil also getting contaminated through faecal shedding of infected domestic and wild animals, and avian species (Gray and Killinger, 1966; Fenlon, 1985) [44, 35].

Moreover, water also acts as a source of infection to cattle (Fenlon, 1999) [33]. In addition, bulk tanks used to store milk were reportedly contaminated with *L. monocytogenes* (Latorre *et al*., 2011) [57]. Presence of *L. monocytogenes* in milk (5.04%) might be due to environmental contamination and the biofilm forming *L. monocytogenes* in milk tank systems.

Manure acts as a good source of nutrient to agricultural soil and thus used as a source of organic fertilizer. However proper curing and ageing of biofertilizers is required before applying it to the soil. By proper treatment, the pathogen counts decline which makes it more useful. Physicochemical conditions such as pH, temperature and slurry dry matter content play an important role affecting the quality of manure (Nicholson *et al*., 2005) [66] It is assumed that the impatient attitude of farmers by using improperly processed manure in agricultural land increases the risk of *L. monocytogenes* contamination to the crop harvest.(Smith *et al*., 2001) [77]. It is important to note that *Listeria* sp. generally survives upto 6 months in dairy slurry; upto 16 days in dairy manure on soil and for 32 days in solid beef (Nicholson *et al*., 2005) [66].

L. monocytogenes was able to grow well in degraded silage with pH on higher side. Similar results have been reported earlier (Fenlon, 1986a [35], Fenlon *et al*., 1989) [32]. *L. monocytogenes* has also been detected from a poor quality silage used to fed the bovines (Low and Renton, 1985) [61]. Similarly the occurance of Listeriosis has been established through silage feeding in bovines (Boerlin *et al.*, 2013) [12].

Silage goes through a rapid anaerobic lactic fermentation by converting sugars into acids which drops the pH (4.5) and thus it got preserved (Fenlon and Shepherd, 2000) [33]. Acidic conditions certainly inhibits the growth of *Listeria* and other spoilage microorganisms (Fenlon and Shepherd, 2000) [33]. However, in presence of aerobic conditions, fermentation starts to drop down and acidic pH may not be retained and hence bacterium like *Listeria* can grow easily (Fenlon and Shepherd, 2000) [33]. *L. monocytogenes* can survive during fermentation and it may multiply rapidly (Kelly *et al*., 2000) [54]. A number of studies reported the poor quality silage act as a source of *L. monocytogenes* transmission to the ruminants (Hinton, 2000, Vela *et al*., 2001) [46, 85]. Further the information is sparsely available on the exact transmission dynamics of *L. monocytogenes* in agriculture production systems. For example, it is not sure how much infection of animals is required for *L. monocytogenes* dispersal into the environment. In the present study, the *L. monocytogenes* subtypes were recorded from clinically affected animals. Its subtypes were also prevalent in environmental samples. Here we observed a clear relationship of *L. monocytogenes* with clinical and environmental samples, as nature remain cross-sectional; Conclusive evidences with exact analysis was difficult to established however it indicate that the animals or bovine farms might be the source of environmental contamination, as *L. monocytogenes* was more dominant in faecal samples compared to the soil samples. The prevalence of *L. monocytogenes* in faecal samples was higher than the feed samples. These results have hypothesized that the animals might exposed to *L. monocytogenes* through contaminated silage which amplified the number of pathogen which in turn resulted in to higher prevalence of *L. monocytogenes* in faeces than in feed. Fenlon (1996) [36] reported that soil samples from silage fed ruminants contaminated pastures possesed higher *L. monocytogenes* count compared to the normal pastures, which refelected that the *L. monocytogenes* strains ingested via silage got amplified and reintroduced into the farm environment through cattle (Fenlon *et al*., 1996) [36].

In the present study, prevalence of *L. monocytogenes* was higher in soil than in feed, which strongly indicated that soil serves as source of animal feed contaminated by *L. monocytogenes*. Similar report earlier been published by Nightangle *et al* (2004) [67] reported prevalence of *L. monocytogenes* in soil and feed (35.21 and 21.36%) respectively.

Transmission of *L. monocytogenes* in domestic ruminants usually occurs through direct faecal shedding (while grazing) or indirectly through manure into agricultural land. In addition, the crops grown on the contaminated soil can directly contaminate the silage (Fenlon *et al*., 1996) [36]. With a heavy load of *L. monocytogenes* in farm environment, contaminated food represents a primary source of this pathogen to human through food chain. It is thus proposed that the bovine hosts could amplify and maintain the high

level of it. *L. monocytogenes* when ingested it through contaminated feeds (Husu, 1990; Vilar *et al*., 2007) [47, 86].

L. monocytogenes has also detected in raw milk which might be due to contamination of milk during its transportation and storage. The *L. monocytogenes* has also been detected earlier in raw milk (Bemrah *et al*., 1998; Frece *et al*., 2010) [10, 39] and thus supported the findings of present study.

In the present study, prevalence of *L. monocytogenes* was recorded as 5.46% in clinical samples from cattle compared to 2.85%.and 3.6% prevalence in the earlier report of (Chopra *et al* 2012, Silva *et al*., 2009) [17, 75] Prevalence of *L. monocytogenes* in milk was reported to be high (3.52% to 0.55%) (Rawool *et al* (2007) [71]; Yadav *et al* (2010) [93]; Gebicova and Karpiskova (2012) [41]. Similarly Mohammad *et al* (2009) [63] recorded 13, 19 and 43% of *L. monocytogenes* from milk, udder swabs and faecal samples of cattle, respectively. Azawi *et al* (2010) [6] reported high prevalence of *L. monocytogenes* (11%) from reproductive tract of Buffaloes and cows from abattoir showing hydrosol pinx. Prevalence of Listeriae in genital and other secretion of animal results in dissemination of organism in the environment which may lead to perpetuation of listeriosis in the farm animals.

In another study Bhilegaonkar *et al* (1997) [11] recorded slightly higher *L. monocytogenes* prevalence of 8.1% among 86 raw milk from northern India. Similarly Barbuddhe *et al* (2002) [9] recorded 6.25% and 26.13% prevalence of *L. monocytogenes* and *Listeria* sp. respectively from buffalo milk (n=64) in Northern India. In a big group survey (n=2060) Kalorey *et al* (2008) [51] recorded about 5.1% of raw milk sample contaminated with *L. monocytogenes* which is in agreement with findings of present study. The prevalence of *L. monocytogenes* in milk samples varied as per geographical areas. In De Manc with 1, 132, 958 raw milk samples tested, the prevalence of *L. monocytogenes* was of 1.2% (Jensen *et al*., 1996) [50], 3.62% in Spain (Gaya *et al*., 1998) [40] and 3.48% in USA (Ryser and Marth, 1991) [72]. In UK, a high incidence of *L. monocytogenes* was recorded from milk processing equipments (18.8%), in environment (54.7%) and in raw milk (22.2%) (Kells and Gilmour, 2004) [53]. This variability in *L. monocytogenes* prevalence in geographical locations might be due to adaptation of diverse isolation and enumeration techniques.

In a number of bovine environments, the prevalence of *L. monocytogenes* was linked with its ability to grow in low temperature along with biofilm formation capability. Few *L. monocytogenes* strains have also been reported to survive beyond the sanitization protocol which made them suitable to grow in an environment (Di Bonaventura *et al*., 2008, Luden *et al*., 2003) [24, 62].

It is reported that cow infection with *L. monocytogenes* were responsible for milk contamination (Winter *et al*., 2004) [90] In addition unhygienic milking and poor barn hygiene are important risk factors to contaminate the milk (Sanaa *et al*., 1993) [73]. As reports of contamination through milk tanks are frequent in countries like Sweden (1%) (Waak *et al*., 2002) [87], United States (4.97%) (Muraoka *et al*., 2003) [65], France (3.2%) and in Mexico (13%) (Vazquez-salinas *et al*., 2001) [84], it poses a concern with shipment. *L. monocytogenes* has also been isolated from animal farm and raw milk collector at dairy level which indicated that every material coming directly or indirectly in contact with milk could transmit the pathogen (Le monnier and Leclercq 2009; Latorre *et al*., 2010) [58, 56]. Many earlier reports highlighted the prevalence of *Listeria* in a range of 4 to 19% in milk and milk processing environment (Fox *et al*., 2009, Almeida *et al*., 2013, Derra *et al*., 2013, Giacometti *et al*., 2013) [38, 2, 21, 43] Bemrah *et al*., (1998) [10]; Kousta *et al*., (2010) [55] reported contamination of milk and milk products by the workers during handling and processing. It is well documented that *L. monocytogenes* contamination in milk does not produce any significant change in appearance due to which most of the Listerial contamination go unnoticed. Moreover the *L. monocytogenes* are capable of biofilm formation making them more potent to surviving and infection if consumed (Harvey *et al*., 2007 [45]; Liu *et al*., 2007) [60].

In the present study, it has concluded that the contamination of *Listeria* sp. in raw milk was mostly due to insufficient hygiene during milking, and storage and transport of milk.

Presence of virulence genes like *iap, prf* A, *plc* A, *hly, mpl, act* A, *plc* B, *Inl* A and *Inl* B in *L. monocytogenes*in from bovine milk\ (Yadav *et al*., 2010) [92], bovine clinical samples (Rawool *et al*., 2007) [71] have been reported Moreever, the *prf*A, *plc*A, *hly*A, *act*A and *iap* among *L. monocytogenes* isolates have also been reported from reproductive disorders in buffaloes (Shakuntala *et al*., 2006) [74] and infected sheep (Yadav and Roy, 2009) [91]

Presence of *hly* A, *actA*, and *Inl*B genes indicated the virulence potential of *L. monocytogenes* to invade and spread intracellularly (Disson and Lecuit 2013 and Travier *et al*., 2013) [25, 82]

The *hly*A gene common to all *L. monocytogenes* isolates. Similar findings were reported by (Aznar and Alarcon, 2002) [7]. The *L. monocytogenes* isolates recovered from different geographical niches showed 92-95% genetic homogenecity (Jaradat *et al;* 2002) [49] and thus corroborates the findings of present study. Moreover the presence of PI-PLC activity along with *hly* A gene confirms the recovery of virulent *L. monocytogenes* strains from different samples in present study.

As per Cheng *et al* (2008) [16] 13 serotypes of *L. monocytogenes* are known, out of which serotypes1/2a, 1/2b and 4b contributes to more than 98% of outbreaks. The present findings showed most of the positive milk, animal clinical samples and bovine environment possessed 1/2b serotype. Similar report has been published by Doumith *et al* (2004) [27] who reported that food borne outbreak generally involve serovar 1/2b. In another study, Fox *et al* (2009) [38] reported presence of 1/2a, 1/2b and 4b serotypes in dairy farm environment, and in milking facility. Recovery of *L. monocytogenes* serotype 1/2a strains along with 1/2b and 4b is responsible for outbreaks in 98% cases as reported in milk processing environments from Brazil where 1/2a was a serotype (Brito *et al*., 2008) [13]. Equal distribution of 1/2b or 3b and 1/2a or 3a seotypes in Portugal dairy has also been reported (Chambel *et al*., 2007) [14].

The serovar 4b dominates in Europe and the serovar 1/2a, 1/2b and 4b in Canada and United States where epidemiological association has been established in perinatal listeriosis with 1/2b and 4b (Gellin *et al*., 1991) [41]. In the present study also common presence of 1/2b, 4b and 1/2a was recorded. In a similar report Singh (2012) [76] sampled *L. monocytogenes* from vegetable, human, milk and observed the 4b serotype especially in milk while the serotype 1/2b in meat product. Serovar 4b has also been associated with obstractics cases in human (Sonegaonkar, 2009) [78].

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) [15, 3, 79]. 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) [69, 93] Depending upon different geographical area, antibiotic resistance patterns of *L. monocytogenes* in food and environmental sources may change (Yan *et al*., 2010) [93]. We tested all *L. monocytogenes* isolates for their antibiotic sensitivity. Interestingly, all the 38 *L. monocytogenes* isolates were resistant to sulphadiazine. The study conducted by Dhanashree *et al.*, (2003) [23] found similar results wherein *L. monocytogenes* isolates which were sensitive towards commonly used antibiotics were reported

Conclusions

The present study showed the prevalence of *L. monocytogenes* in clinical bovines and their environment from Nagpur region of Maharashtra, India. It has been suggested that there may be a link between serotype and virulence potential The epidemiological studies would help to understand the sources of infection and their risk assessment, routes of transmission, clinical forms and allowed them for better management of the listerial infection. In conclusion this study with high prevalence of *L. monocytogenes* on farms provides additional support for the role of ruminant farms as reservoirs for this food borne pathogen.

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References

1. Adhikary R, Joshi S. Neonatal listeriosis: A case report from sub-Himalayas. Indian. J Med. Microbiol. 2010; 29(1):79.
2. Almeida G, Magalhaes R, Carneiro L, Santos I, Silva J, Ferraira V *et al*. Foci of contamination *Listeria monocygones* in different cheese processing plants. Int. J Food Microbiol. 2013; 167:303-309.
3. Altuntas EG. Antibiotic and bacteriocin sensitivity of *Listeria monocytogenes* strains isolated from different foods. Food Nutri. Sci. 2012; 3(3):363-368.
4. Anon. Performance Standards for Antimicrobial Susceptibility Testing, Sixteenth Informational Supplement, M100-S16. Clinical and Laboratory Standard Institute. 2006; 26(3).
5. Atil E, Ertas HB, Ozbey G. Isolation and molecular characterization of *Listeria* spp. from animals, food and environmental samples. Veterinarni Medicina. 2011; 56(8):386-394.
6. Azawi OI, Al-Abidy HF, Ali AJ. Pathological and bacteriological studies of hydrosalpinx in buffaloes. Reprod Domest Anim. 2010; 45(3):416-20.
7. Aznar R, Alarcón B. On the specificity of PCR detection of *Listeria monocytogenes* in food: a comparison of published primers. Syst. Appl. Microbiol. 2002; 25:109-119.
8. Barbuddhe SB, Hain T, Chakraborty T. The Genus Listeria in Practical handbook of Microbiology. CRC Press, Boca Raton, 2008 533-562.
9. Barbuddhe SB, Chaudhari SP, Malik SVS. The occurrence of pathogenic *Listeria monocytogenes* and antibodies against Listeriolysin O in buffaloes. J Vet. Med. B., 2002; 49:181-184.
10. Bemrah N, Sanaa M, Cassin MH, Grifiths MW, Cerf O. Quantitative risk assessment of human listeriosis from consumption of soft cheese made from raw milk. Prevent. Vet. Med. 1998; 37:129-145.
11. Bhilegaonkar KN, Kulshresta SBM, Kapoor KN, Kumar A, Agarwal RK, Singh BR. FAO/WHO collaborating centre for research, Izatnagar Training in Veterinary public health. Indian Veterinary Research Institute, Izatnagar. 1997, 348-250.
12. Boerlin P, Boerlin- Petzold F, Thomas J. Use of Listeriolysin O and Internalin A in a seroepidemiological study of Listeriosis in Swiss Dairy Cow. J Clini. Microbial. 2013, 1055-1061.
13. Brito JR, Santos EM, Arcuri EF, Lange CC, Brito MA, Souza GN *et al*. Retail survey of Brazilian milk and Minas frescal cheese and a contaminated dairy plant to establish prevalence relatedness and sources of *Listeria monocytogenes* isolates. Appl. Environ Microbial. 2008; 74(15):4954-4961.
14. Chambel L, Sol M, Fernandes I, Barbosa M, Zilhao I, Barata B *et al*. Occurrence and persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal unveiled by an integrated analysis of identification, typing and spatial-temporal mapping along production cycle Int. J of Food Microbiol. 2007; 116: 52-63.
15. Charpentier E, Courvalin P. antibiotic resistance in *Listeria* spp. Antimicrob Agents. Chemother. 1999; 43(9):2103-2108.
16. Cheng Y, Robin S, Kathariou S. Genomic Division/Lineages, Epidemic clones, and Population Structure. Handbook of *Listeria monocytogenes*, CRC press, Boca Raton. 2008, 337-358.
17. Chopra S, Sharma V, Shukla S, Nayak A. Antibiogram of *Listeria* spp. Isolate from reproductive disorders and livestock products of ruminants. Journal of Animal Research. 2012; 2(2):87-190.
18. Cowan ST, Steel KJ. Manual for the identification of Medical Bacteria. The syndics of the Cambridge University Press. Bentlay House, 200 Euston Road, London, U.K, 1993.
19. Cruickshank R, Duguid JP, Marimon BP, Swain BP. Medical Microbiology, 12th edn., Churchill Livingstone, Edinburg, London, 1975.
20. Deb R, Kumar A, Chakraborty S, Verma AK, Tiwari R, Dhama K *et al*. Trends in diagnosis and control of bovine mastitis: A review. Pak. J Biol Sci. 2013; 16(23):1653-1661.
21. Derra FA, Karlsmose S, Monga DP, Mache A, Svendsen CA, Felix B *et al*. Occurrence of *Listeria* spp. in retail meat and dairy products in the area of Addis Ababa, Ethiopia. Foodborne Pathog Dis. 2013; 10:577579.
22. Dhama K, Verma AK, Rajagunalan S, Kuma A, Tiwari R, Chakraborty S *et al*. *Listeria monocytogenes* infection in poultry and its public health importance with special reference to food borne zoonoses. Pak. J Biol. Sci. 2013; 16(7):301-308.
23. Dhanashree B, Otta SK, Karunasagar I, Goebel W, Karunasagar I. Incidence of *Listeria* spp. in clinical and food samples in Mangalore, India. Food. Micobiol. 2003;

20(4):447-453.
24. Di Bonaventura G, Piccolomini R, Paludi D, D'Orio V, Vergara A, Conter M *et al*. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. J. App. Microbiol. 2008; 104(6):1552-61.
25. Disson O, Lecuit M, *In vitro* and *in vivo* models to study human listeriosis: mind the gap. Microbes Infect. 2013; 15:971-80.
26. Donnelly CW, Baigent GJ. Methods for flow cytometric detection of *Listeria monocytogenes* in milk. Appl. Environ. Microbiol. 1986; 52(4):689-695.
27. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin Microbiol. 2004; 42(8):3819-3822.
28. EPA (Environmental Protection Agency) Office of Waste-water Enforcement and Compliance. Guide to field storage of biosolids. Report 832-B-00-007. 2000a.Avail able at www.epa.gov/owmitnet/mtb/biosoli ds/fsguide/ fsg uide.pdf. Accessed 30 October 2006.
29. EPA (Environmental Protection Agency) Office of Water Proposed regulations to address water pollution from concentrated animal feeding operations, 2000b. Report 833-F-00-016. 2000b.Available at www.p2pays.org/r ef/01/00830.pdf. Accessed 30 October 2006.
30. Farber JM, Peterkin PI *Listeria monocytogenes*, a food borne pathogen Microbiol Rev. 1991; 55(3):476-511.
31. Fenlon DR. *Listeria monocytogenes* in the natural environment, InE. T. Ryser and E. H. Marth (ed.), Listeria, listeriosis and food safety, 2nd ed. Marcel Decker, Inc., New York, N.Y. 1999, 21-38.
32. Fenlon DR, Wilson J. The incidence of *Listeria monocytogenes* in raw milk from farm bulk tanks in North- East Scotland. J Appl. Microbiol. 1989; 66(3):191-196.https://doi.org/10.1111/j.1365-2672.1989.tb02469.
33. Fenlon DR, Shepherd JL. Ecology of *Listeria monocytogenes*: Studies on Incidence, Growth, and Microbial competition in Primary Production. Proceedings from the ILSI North America Symposium Series on Food Microbiol. 2000, 26-28.
34. Fenlon DR. Wild birds and silage as reservoirs of Listeria in the agricultural environment. J. Appl Bacteriol 1985; 59:537-543.
35. Fenlon DR. Rapid quantitative assessment of the distribution of Listeria in silage implicated in a suspected out-break of listeriosis in calves. Vet Rec. 1986a; 118:240-242.
36. Fenlon DR, Wilson J, Donachie W. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. J Appl Bacteriol. 1996; 81:641-650.
37. Fenlon DR. Rapid quantitative assessment of the distribution of Listeria in silage implicated in a suspected outbreak of listeriosis in calves. Vet. Rec. 1986; 118:240-242.
38. Fox E, O' Mahony T, Clancy M, Dempsey R, O'Brien M, Jordan K. *Listeria monocytogenes* in the Irish dairy farm environment. J Food Prot. 2009; 72:1450-1456.
39. Frece J, Markov K, Cvek D, Kolarec K, Delas F. Comparison of conventional and molecular methods for the routine confirmation of *Listeria monocytogenes* in milk products produced domestically in Croatia. J. Dairy Res. 2010; 77:112-116.
doi: 10.1017/S0022029909990379.
40. Gaya P, Sanchez R, Medina M, Nunez M. Incidence of *Listeria monocytogenes* and other *Listeria species* in raw milk produced in spain. Food Microbiol. 1998; 15:551-555.
41. Gelbicova T, Karpiskova R. Outdoor environment as a source of *Listeria monocytogenes* in food chain. Czech J Food Sci. 2012; 30(1):83-88.
42. Gellin BG, Broome CV, Broome WF, Bibb WF, Weaver RE, Gaventa SL. Mascola and the Listeriosis study group The epidemiology of listeriosis in the United States – 1986. Am. J Epidemiol. 1991; 133:392-401.
43. Giacometti F, Bonilauri P, Serraino A, Bolzoni G. Four – year Monitoring of food borne pathogens in raw milk sold by vending Machines in Italy. J of Food Prot. 2013; 76(11):1902-1907.
44. Gray ML, Killinger AH. *Listeria monocytogenes* and listeric infection. Bacteriol Rev 1966; 30:308-382.
45. Harvey J, Keenan KP, Gilmour A. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiol. 2007; 24:380-392.
46. Hinton MH. Infections and intoxications associated with animal feed and forage which may present a hazard to human health. Vet. J. 2000; 159:121-128.
47. Husu JR. Epidemiological studies on the occurrence of *Listeria monocytogenes* in the feces of dairy cattle. J Vet. Med. Ser. 1990; B37:276-282.
48. Jallewar PK, Kalorey DR, Kurkure NV, Pande VV, Barbuddhe SB. Genotypic characterization of *Listeria spp*. isolated from fresh water fish. Int. J Food Microbiol. 2007; 114(1):120-123.
49. Jaradat ZW, Schutze GE, Bhunia AK. Genetic homogenecity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ri botyping and PCR. Int. J Food Microbiol. 2002; 76(1, 2):1-10.
50. Jensen NE, Aerestrup FM, Jensen J, Wegener HC. *Listeria monocytogenes* in bovine mastitis. Possible implication for human health. Int. J Food Microbiol. 1996; 32:209-216.
51. Kalorey DR, Kurkure NV, Warke SR, Rawool DB, Barbuddhe SB. *Listeria* species in bovine raw milk: A large survey of Central India. Food Cont. 2008; 19(2):109-112.
52. Kanauchi T, Kobayashi M, Tsuruta M, Sobu H, Niizeki H, Hayasaka K. Listeriosis in a dairy cow and simultaneous isolation of the causative agent from silage. J Jpn. Vet. Med. Assoc. 1987; 40:850-853.
53. Kells J, Gilmour A. Incidence of *Listeria monocytogenes* in two milk processing evironments and assessment of *Listeria monocytogenes* blood agar for isolation. Int. J. Food Microbiol. 2004; 91:167-174
54. Kelly LA, Gibson G, Gettinby G, Donachie W, Low JL. A predictive model of the extent of listerial contamination with in damaged silage bales. Quant. Microbiol. 2000; 2:171-188.
55. Kousta MM, Mataragas, Skandamis P, Drosinos EH. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. Food Control. 2010; 21(6):805-815.
http:// dx.doi.org/10.1016/j.foodcont.2009.11.015.

56. Latorre A, VanKessel J, Karns J, Zurakowski M, Pradhan A, Boor K *et al*. Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. J Dairy Sci. 2010; 93(6):2792-802.
57. Latorre A, Van Kessel J, Karns J, Zurakowski M, Pradhan A, Boor K *et al*. Increased *in vitro* adherence and on-farm persistence of predominant and persistent *Listeria monocytogenes* strains in the milking system. Appl. Environ. Microbiol. 2011; 77(11):3676-84.
58. Le Monnier A, Leclercq A. Listeria and Listeriosis from farm to frok.Pathol. Biol. 2009; 57(1):17-22.
59. Leite P, Rodriguez R, Ferreira M, Ribeiro G, Jacquet C, Martin P *et al*. Comparative characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from humans. Int. J of Food Microbiology. 2006; 106:111-121.
60. Liu D. Epidemiology in Handbook of *Listeria monocytogenes*. CRC press, Boca Raton, 2008, 27-60.
61. Low JC, Renton CR. Septicemia, encephalitis and abortion in housed flock of sheep caused by *Listeria monocytogenes*. Vet. Res. 1985; 116:147-150.
62. Lunden JM, Autio TJ, Sjoberg AM, Korkeala HJ. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. J Food Prot. 2003; 66:2062-2069.
63. Mohammed HO, Stipetic K, McDonough PL, Gonzalez RN, Nydam DV, Atwill ER. Identification of potential on-farm sources of *Listeria monocytogenes* in herds of dairy cattle. Am J Vet Res. 2009; 70(3):383-8.
64. Moharem AS, Raj APC, Janardhana GR. Incidences of *Listeria species* in seafood of Mysore, India. J Food Safety. 2007; 27(4):362-372.
65. Muraoka W, Gay C, Knowles D. Prevalence of *L. monocytogenes* subtypes in bulk tank milk of the Pacific Northwest. J Food Prot. 2003; 66:1413-1419.
66. Nicholson FA, Groves SJ, Chambers BJ. Pathogen survival during livestock manure storage and following land application. Bioresour Technol. 2005; 96:135-143.
67. Nightingale KK, Schukken YH, Nightingale CR, Fortes ED, Ho AJ, Her Z *et al*. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the Farm environment. Appl. And environmental Microbiology. 2004, 4458-4467.
68. Notermans SHW, Dufrenne J, Leimeister-Wachter M, Domann E, Chakraborty T. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. Al. Envirol. Microbiol. 1991; 57:2666-2670.
69. Palumbo, Daniela, Iannaccone, Marco Porta, Amalia, Capparelli, Rosanna. Experimental antibacterial therapy with puroindolines, lactoferrin and lysozyme in *Listeria monocytogenes*-infected mice. Microbes and infection / Institut Pasteur. 2010; 12:538-45. 10.1016/j.micinf.2010.03.010.
70. Pritchard TJ, Donnelly CW. Combined secondary enrichment of primary enrichment broths increases Listeria detection. J of Food Protection. 1999; 62:532-535.
71. Rawool DB, Malik SVS, Shakuntala I, Sahare AM, Barbuddhe SB. Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases. Int. J Food Microbiol. 2007; 113:201-207.
72. Ryser ET, Marth EH. Listeria, Listeriosis and food safety Marcel Dekker, New York (Basel), 1991
73. Sanaa M, Poutrel B, Menard JL, Serieys F. Risk factors associated with contamination of raw milk by *Listeria monocytogenes* in dairy farms. J Dairy Science. 1993; 76:2891-2898.
74. Shakuntala I, Malik SVS, Barbuddhe SB, Rawool DB. Isolation of *Listeria monocytogenes* from buffaloes with reproductive disorders and its confirmation by polymerase chain reaction. Vet. Microbiol., 2006; 117:229-234.
75. Silva TMA, de Oliveira RG, Da Silva Mol JP, Xavier MN, da Paixão TA, Cortez A *et al*. Etiologic diagnosis of bovine infectious abortion by PCR. Ciência Rural, Santa Maria. 2009; 39(9):2563- 2570.
76. Singh UA. Random Amplified Polymorphic DNA (RAPD) analysis of *Listeria monocytogenes.* M.V.Sc thesis submitted to MAFSU, Nagpur, 2012.
77. Smith KA, Brewer AJ, Dauven A, Wilson DW. A survey of the production and use of animal manures in England and Wales. III. Cattle manures. Soil Use Manage. 2001; 17:77-87.
78. Sonegaonka AS. Molecular Characterization of *L. monocytogenes* of human and animal origin. M.V.Sc. Thesis submitted to Maharashtra Animal & Fishery Sciences University, Nagpur, 2009.
79. Soni DK, Singh RK, Singh DV, Dubey SK. Characterization of *Listeria monocytogenes* isolated from Ganges water, human clinical and milk samples at Varanasi, India. Infect. Genet. Evol. 2013; 14:83-91.
80. Suarez, Vazquez Boland. The bacterial actin nucleated protein ACTA is involved in epithelial cell invasion by *Listeria monocytogenes*, 2001. vailable at http://www.ncbi.nim.nih.gov/pubmed (Accession No. AF103807).
81. Swaminathan B, Cabanes D, Zhang W, Cossart P. *Listeria monocytogenes*. In M.P. Doyle, and L.R. Beuchat (Eds.). Food Microbiology: fundamentals and frontiers (3rd ed.) Washington D.C.; ASM Press, 2007.
82. Travier L, Guadagnini S, Gouin E, Dufour A, Chenal-Francisque V, Cossart P, *et al*. *Act*A Promotes *Listeria monocytogenes* Aggregation, Intestinal Colonization and Carriage. PLoS Pathog, 2013; 9:e1003131
83. Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W *et al*. *Listeria* Pathogenesis and Molecular Virulence Determinants. Clin.Microbiol. Rev. 2001; 14(3):584-640.
84. Vázquez-Salinas C, Rodas-Suárez O, Quiñones-Ramirez EI. Occurrence of *Listeria species* in raw milk in farms on the outskirts of Mexico city. Food Microbiol. 2001; 18(2):177-181.
85. Vela AI, Fernandez-Garayzabal JF, Vazquez JA, Latre MV, Blanco MM, Moreno MA *et al*. Molecular typing by pulsed-field gel electrophoresis of Spanish animal and human *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 2001; 67:5840-5843.
86. Vilar MJ, Yus E, Sanjuan MC, Dieguez FJ, Rodriguez JL. Prevalence and risk factors for *Listeria* spp. On dairy farms. J dairy Sci. 2007; 90(11):5083-5088.
87. Waak E, Tham W, Danielsson-Tham ML. Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm bulk tanks and dairy plant receiving tanks. Appl Environ Microbiol. 2002; 68:3366-3370.

88. Weis J, Seeliger HPR. Incidence of *Listeria monocytogenes* in nature Appl. Microbiol. 1975; 30:29-32.
89. Welshimer HJ. Survival of *Listeria monocytogenes* in soil. J Bacteriol. 1960; 80:316-320.
90. Winter P, Schilcher F, Bago Z, Schoder D, Egerbacher M, Baumgartner W *et al*. Clinical and histopathological aspects of naturally occurring mastitis caused by *Listeria monocytogenes* in cattle and ewes. Zoonosis and Public Health. 2004; 51(4):176-179
91. Yadav MM, Roy A. Prevalence of *Listeria* spp Including *Listeria monocytogenes* from Apparently Healthy Sheep of Gujarat State, India. Zoonosis and Public Health. 2009; 56(9, 10):515-524.https://doi.org/ 10.1111/j.1863-2378.2008.01201
92. Yadav MM, Roy A, Bhanderi B, Joshi C. Pheno-genotypic characterization of *Listeria monocytogenes* from bovine clinical mastitis. Buffalo Bulletin. 2010; 29:29-38
93. Yan H, Neogi SB, Mo Z, Guan W, Shen Z, Zhang S *et al*. Prevalence and characterization of antimicrobial resistance of foodborne Listeria monocytogenes isolates in Hebei province of Northern China, 2005 – 2007. International Journal of Food Microbiology. 2010; 144(2):310-316. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.10.015