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Molecular characterization of *Listeria monocytogenes* in bovine milk and evaluating the sensitivity of PCR for direct detection in milk

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ABSTRACT

Food-borne listeriosis, recognized as an emerging bacterial disease of humans and animals worldwide, is caused by *L. monocytogenes* with at least 95% of the strains isolated from foods and patients belonging to serovars 1/2a, 1/2b and 4b. Milk and dairy products were implicated as sources of listeriae in several widely publicized incidents, thus suggesting that the mammary glands of mastitic cattle may be an important reservoir of *Listeria*. In the present study, 350 bovine milk samples were collected for prevalence and molecular characterization studies of *Listeria* spp. The isolates were phenotypically and genotypically characterized by biochemical tests, haemolysis on sheep blood agar, CAMP test, PI-PLC assay and multiplex PCR targeting virulence cluster genes namely haemolysin (*hly*A), PI-PLC (*plc*A), actin (*act*A), p60 (*iap*) and regulatory (*prf*A); along with multiplex PCR for typing major serovars targeting *lmo0737,* ORF2819**,** ORF2110 and *prs* genes. Four pathogenic *L. monocytogenes* were recovered indicating prevalence rate of 1.14% in milk while the overall prevalence rate of *Listeria* spp. was 1.42%. All the four pathogenic isolates were characterized as *L. monocytogenes* serotype 4b. Antibiogram of the pathogenic *L. monocytogenes* isolates revealed sensitivity for amikacin, gentamycin, norfloxacin and doxycyclin. Animal sera (169) screened by indirect ELISA for antibodies against listeriolysin O showed sero-positivity of 7.1%. Sensitivity of PCR for direct detection from milk was evaluated to be 8.8×10^5 *L. monocytogenes* cells/ml of milk. Thus, the presence of pathogenic strains of *L. monocytogenes* in raw milk appeared to be a cause for concern with profound public health implications.

Key words: Antibiogram, Bovine, ELISA, *Listeria monocytogenes*, Listeriosis, Milk, Sensitivity, Serotype

Listeriosis, an important food-borne infection, is caused by *Listeria monocytogenes*. Till date, 13 serovars have been described for the species *L. monocytogenes*, at least 95% of the strains isolated from foods and patients were of serovars 1/2a, 1/2b and 4b (Graves *et al*. 1999). Various reports indicate that *Listeria* spp. can be found in dairy products (Marth and Ryser 1990), but the outbreaks associated with the consumption of milk (Brito *et al*. 2008) are of great concern in the dairy industry due to high mortality rate. One of the reasons for infection via dairy products could be attributed to the ability of *L. monocytogenes* to survive and grow over a wide range of temperature and low pH (Gahan *et al.* 1996).

The pathogen was found involved in numerous outbreaks of listeriosis involving the consumption of milk and milk products (Jamali *et al.* 2013, Ning *et al.* 2013). India, being the largest producer of milk in the world, also has the highest number of cattle in the world. During the year 2011–12, the estimates of milk production revealed the milk production in Punjab as 9.55 million tonnes versus 127.904 million tonnes for India (Source: Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India). Various workers in India have also reported the isolation of pathogenic *L. monocytogenes* from milk (Bhilegaonkar *et al.*1997, Barbuddhe *et al.* 2002, Rawool *et al.* 2007). Current microbiological culture methods require at least 5 days to isolate *Listeria* colonies from food matrices and additional days for the identification at the species level, and are therefore time-consuming and laborious (Rapporti ISTISAN 1996). PCR-based detection systems, which are specific and sensitive, were thus proposed to eliminate the enrichment and culturing step (Makino *et al.* 1995). The present study, therefore is aimed to undertake the phenotypic and molecular characterization of *L. monocytogenes* isolates identified from milk samples of cows and buffaloes in Punjab state of Northern India and to evaluate the sensitivity of PCR for direct detection of *L. monocytogenes* in milk, thereby eliminating the need for enrichment and subsequent culturing on plating media.

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MATERIALS AND METHODS

Bacterial cultures: The standard strains of *Listeria monocytogenes* 4b (ATCC 19115), *Staphylococcus aureus* (ATCC 11632) and *Rhodococcus equi* (ATCC 6939) were used in this study.

Samples: Bovine milk samples (350) were collected

aseptically in sterilized vials from organized and unorganized dairy farms from different regions of Punjab state of North India. In addition to these, 169 serum samples were collected from some of these animals to assess the seroprevalence. After labelling, the samples were immediately transported to laboratory in ice box maintaining cold chain, where after separation of serum from blood, samples were stored at –20°C in deep freeze till further analysis.

Isolation of Listeria: Isolation of *Listeria* from the milk and blood samples of animal and hand swabs of the farm workers was attempted as per McClain and Lee (1988) after making necessary modifications.

Confirmation of the isolates: Morphologically typical colonies of *Listeria* were picked (at least 3/plate) and verified by Gram's staining, catalase reaction, tumbling motility at 25°C, methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol, lactose, glucose and α-methyl-d-mannoside), phosphatidylinositol-specific phospholipase C (PI-PLC) activity on Agar *Listeria* according to Ottaviani and Agosti (ALOA), haemolysis on 7% sheep blood agar and CAMP test with *Staphylococcus aureus* and *Rhodococcus equi* (Seeliger and Jones 1986).

Antibiotic susceptibility test: Antibiotic sensitivity of *Listeria* isolates to various antibiotics and chemotherapeutic agents was studied by agar disc diffusion method using single antibiotic disc (Bauer *et al.* 1966).

ELISA: Indirect plate ELISA was performed for detection of antibodies against listeriolysin O (ALLO). Briefly, polyvinyl microtitre plates were coated with 100µl carbonate buffer (pH 9.6) and purified LLO as an antigen in a concentration of 5,000 ng/ml was added. The plates were incubated at 37°C for 2 h. The coated plates were washed four times with PBST in order to remove unadsorbed antigen.

The unsaturated sites of the plates were blocked by adding 5% skimmed milk powder prepared in PBS @ 200µl /well and incubated at 4°C overnight. The plates were washed thrice with PBS-T. The serum was diluted in range of 1:100 to 1:800 and each dilution was added @100 µl/ well and plates were further incubated at 37°C for 2 h. The plates were washed thrice with PBS-T and added with anti-species HRPO conjugate in the range of 1:5,000 to 1:10,000 and again incubated at 37°C for 2 h.

The plates were washed thrice with PBS-T and finally added with freshly prepared substrate solution. The plates were incubated in dark for 15 min and the colour developed was measured by ELISA reader at 492 nm wavelength. In the standardized ELISA employing LLO (5,000 ng/ml of coating buffer) as an antigen and anti-species HRPO conjugate at 1:5,000 dilution, serum sample at the dilution of 1:100 with the positive-to-negative (P/N) ratio of > 2 was considered as positive for the presence of anti-LLO antibodies.

Polymerase chain reaction: DNA extraction of *L. monocytogenes* was performed as per manufacturer's instructions and used as template in multiplex PCR .

Polymerase chain reaction for typing of major serovars: The multiplex PCR targeting genes namely *lmo0737*, ORF2819, ORF2110 and *prs* for the major *L. monocytogenes* serovars like 1/2a, 1/2b and 4b was standardized employing primers as per Doumith *et al.* (2004) (Table 1).

Based on optimization trials, the standardized PCR protocol for 50µl reaction mixture included 5.0µl of 10 '×' PCR buffer, 4µl of 10 mM dNTP mix (a final concentration of 8 mM), 6µl of 25 mM $MgCl_2$ (a final concentration of 3 mM) and 0.6µl of 100 µM of a primer set containing forward and reverse primers (a final concentration of 1.2 µM of each primer), 1 unit of Taq DNA polymerase, 2µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The DNA amplification was performed in gradient thermocycler with a pre-heated lid. The PCR cycling conditions included an initial denaturation of DNA at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 45 sec and extension at 72°C

Table 1. Details of primers used for amplification of PCR targeting genes for *Listeria monocytogenes* serovars (Doumith *et al.* 2004)

Primer names	Primer sequence		Product size	Serovar specificity
lmo0737	Forward	5'- AGG GCT TCA AGG ACT TACCC - 3'	691 bp	*L. monocytogenes* serovars 1/2a, 1/2c, 3a and 3c
	Reverse	5'- ACG ATT TCT GCT TGC CAT TC - 3'		
lmo1118	Forward	5'- AGG GGT CTT AAA TCC TGG AA- 3'	906 bp	*L. monocytogenes* serovars 1/2c and 3c
	Reverse	5'- CGG CTT GTT CGG CAT ACT TA- 3'		
ORF2819	Forward	5'- AGC AAA ATG CCA AAA CTC GT - 3'	471 bp	*L. monocytogenes* serovars 1/2b, 3b, 4b, 4d, and 4e
	Reverse	5'- CAT CAC TAA AGC CTC CCA TTG - 3'		
ORF2110	Forward	5'- AGT GGA CAA TTG ATT GGT GAA - 3'	597 bp	*L. monocytogenes* serovars 4b, 4d, and 4e
	Reverse	5'- CAT CCA TCC CTT ACT TTG GAC - 3'		
prs	Forward	5'- GCT GAA GAG ATT GCG AAA GAA G - 3'	370 bp	All *Listeria* spp.
	Reverse	5'- CAA AGA AAC CTT GGA TTT GCG G - 3'		

Table 2. Details of primers used for amplification of virulence marker associated genes of listeriae

Primer names	Primer sequence		Product size	Reference
plc A	Forward	5'- CTG CTT GAG CGT TCA TGT CTC ATC CCC C - 3'	1484 bp	Notermans *et al.* (1991)
	Reverse	5'- CAT GGG TTT CAC TCT CCT TCT AC - 3'		
act A	Forward	5'- CGC CGC GGA AAT TAA AAA AAG A - 3'	839 bp	Suarez and Vazquez-Boland (2001)
	Reverse	5'- ACG AAG GAA CCG GGC TGC TAG - 3'		
hly A	Forward	5'- GCA GTT GCA AGC GCT TGG AGT GAA - 3'	456 bp	Paziak-Domanska *et al*. (1999)
	Reverse	5'- GCA ACG TAT CCT CCA GAG TGA TCG - 3'		
iap	Forward	5'- ACA AGC TGC ACC TGT TGC AG - 3'	131 bp	Furrer *et al*. (1991)
	Reverse	5'- TGA CAG CGT GTG TAG TAG CA -3'		
prf A	Forward	5'- CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG - 3'	1060 bp	Notermans *et al.* (1991)
	Reverse	5'- AGC AAC CTC GGT ACC ATA TAC TAA CTC - 3'		

for 45 sec, followed by a final extension of 2 min at 72°C and hold at 4°C. All the 4 sets of primers for serotyping associated genes were amplified under the similar PCR conditions and amplification cycles. The resultant PCR products were further analyzed by agarose gel electrophoresis.

Multiplex polymerase chain reaction for the detection of virulence cluster genes: The multiplex PCR was standardized for the detection of virulence cluster genes, namely haemolysin (*hly*A), PI-PLC (*plc*A), actin (*act*A), p60 (*iap*) and regulatory (*prf*A) by following the method of Rawool *et al.* (2007a) with suitable modifications using standard strain of *L. monocytogenes* 4b (ATCC 19115) (Table No. 2).

The standardized PCR protocol for 50µl reaction mixture consisted of 5.0µl of 10× PCR buffer, 4µl of 10 mM dNTP mix (a final concentration of 8 mM), 4µl of 25 mM $MgCl_2$ (a final concentration of 2 mM) and 0.4µl of 100 µM of a primer set containing forward and reverse primers (a final concentration of 8 µM of each primer), 1 unit of Taq DNA polymerase, 2µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The cycling conditions for PCR included an initial denaturation of DNA at 94°C for 2 min followed by 35 cycles each of 30 sec denaturation at 94°C, 1 min of annealing at 53°C and 1 min 30 sec extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The resultant PCR products were further analyzed by agarose gel electrophoresis.

Sensitivity of the PCR (minimum detection level): The standardized PCR was assessed for its sensitivity to directly detect *L. monocytogenes* from milk. Briefly, *Listeria monocytogenes* 4b (ATCC 19115) culture was grown overnight in BHI broth and centrifuged at 6,000 rpm for 10 min and the pellet obtained was washed once with phosphate buffered saline (PBS), pH 7.2.

Nine tubes, each containing 9 ml of commercially available pasteurized cow milk, which was culturally negative for *Listeria* were taken. Milk sample present in Tube No. 1 was spiked with 1 ml of the pellet, followed by 10–fold serial dilutions till Tube No. 9. Standard plate count (SPC) was done in triplicates per dilution and plates were kept for incubation at 37°C for 24 h. The values of SPC ranged from 8.8×10^7 to 8.8×10 cells/ml in different dilutions inoculated with *L. monocytogenes* cells at serial concentrations. Aliquots (1ml) from each dilution of the spiked milk samples were centrifuged at 12,000 rpm for 10 min. The pellet obtained was washed and dissolved in sterilized milliQ water (100µl) and subjected to DNA extraction. The template DNA was prepared and PCR was performed by targeting *hly*A gene using conditions described as above and the product was analyzed by agarose gel electrophoresis. Sequencing of the *Listeria* isolates were carried out and submitted to Gene bank with accession numbers as KP965732 and KP965733.

RESULTS AND DISCUSSION

The microbiological and biochemical analysis of 350 milk samples from bovines (cattle and buffaloes) identified 5 isolates revealing typical characteristics *Listeria* spp. Out of these, 4 isolates were characterized as *L. monocytogenes,* while the remaining one was identified as *L. grayi*. The overall occurrence of *Listeria* spp. turned out to be 1.42%.

The present finding can be supported by the findings of Rawool *et al.* (2007b) who reported 1.66% isolation rate of *Listeria* spp. from cases of clinical bovine mastitis in the Uttar Pradesh. However, Kalorey *et al.* (2008) reported 5.1% prevalence of *L. monocytogenes* from dairy cows. More recent studies from India have reported prevalence of 3.52% of *L. monocytogenes* from mastitic milk samples (Yadav *et al.* 2010), while Soni *et al.* (2013) reported 5.8% prevalence of *L. monocytogenes* in cow milk samples collected from Varanasi, Uttar Pradesh, India.

On pathogenicity testing of the *Listeria* isolates of the present study by the PI-PLC assay, haemolysis on 7% sheep blood agar and CAMP test, all *L. monocytogenes* isolates were found pathogenic, whereas *L. grayi* isolate was negative for all the tests. The isolation of pathogenic *L. monocytogenes* from milk revealed an overall prevalence of 1.14%.

L. monocytogenes isolates when subjected to multiplex PCR for serotyping were found to amplify the DNA fragments of 471, 597 and 370 bp, respectively, indicating *L. monocytogenes* serotype 4b (Fig.1).

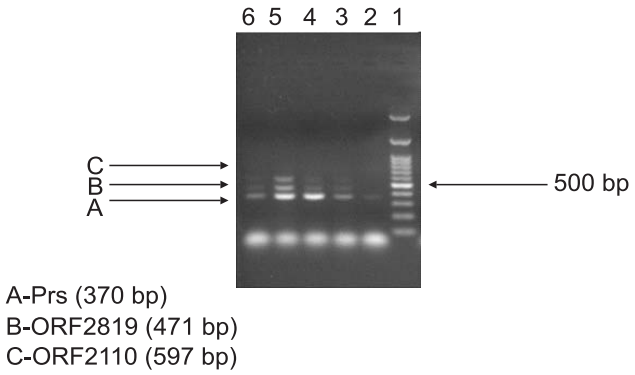

Fig. 1. PCR profile of serovar associated genes in standard *L. monocytogenes.* Lane 1: PCR marker (100 to 3,000 bp); Lane 2: Amplified products of 1 gene i.e. *prs* (370 bp) (which was found to be *L.* GRAYI); Lane 3: Lane 4, Lane 5 and Lane 6: Amplified products of 3 genes i.e. *prs* (370 bp), ORF2819 (471 bp), ORF2110 (597 bp).

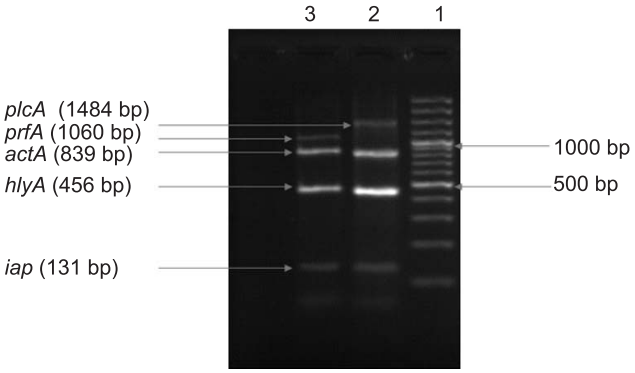

Fig. 2. PCR profile of virulence associated genes in standard *L. monocytogenes.* Lane 1: PCR marker (100 to 3,000 bp); Lane 2: Amplified products of 4 genes i.e. *plcA* (1484 bp), *actA* (839 bp), *hlyA* (456 bp), *iap* (131bp); Lane 3: Amplified products of 4 genes i.e. *prfA* (1060 bp), *actA* (839 bp), *hlyA* (456 bp), *iap* (131 bp).

L. monocytogenes isolates exhibited presence of all the virulence associated genes in multiplex PCR (Fig 2), thus confirming their pathogenic nature as briefed in Table No. 3. The findings in the present study are in agreement with the work carried out by Rawool et al. (2007) who reported pathogenic isolates of *L. monocytogenes* recovered from bovine milk, which showed the presence of *plc*A, *act*A, *hly*A, *prf*A and *iap* genes. Yadav *et al.* (2010) isolated *L. monocytogenes* from bovine mastitic milk samples from Gujarat, which by PCR confirmed the presence of *plc*A, *prf*A, *hly*A, *act*A and *iap* genes.

In the present investigation, a study of PCR profiles of 4 *L. monocytogenes* isolates showed a positive correlation and co-expression of *prf*A, the master regulator, which is directly involved in the control of virulence gene expression (Chakraborty *et al.* 1992).

All *L. monocytogenes* isolates possessed *plc*A gene as well as expressed PI-PLC activity in the agar. This observation is in agreement with that of other workers who reported that only the pathogenic strains of *L. monocytogenes* possess PI-PLC (*plc*A) gene and only

Table 3. Biochemical and pathogenicity profiles of *Listeria* isolates

Sr. no.	Isolate code	Biochemical profile						Species identified	Pathogenicity profile					
		Rhamnose	Xylose	α-Methyl-D-Mannoside	Mannitol	Hemolysis on SBA	CAMP with S/R		*In vitro* test					
									PI-PLC assay		PCR profile of serovar-associated genes			
									colour	*Halo*	*plcA*	*hlyA*	*actA*	*iap*	*prfA*
01	LM 1	+	-	+	-	+	+S	*L.m.*	BG	+	+	+	+	+	+
02	LM 2	+	-	+	-	+	+S	*L.m.*	BG	+	+	+	+	+	+
03	LM 3	+	-	+	-	+	+S	*L.m.*	BG	+	+	+	+	+	+
04	LM 4	+	-	+	-	+	+S	*L.m.*	BG	+	+	+	+	+	+
05	LG 1	+	-	+	+	-	-	*L.g*	BG	-	-	-	-	-	-

*L.*m., *L. monocytogenes;* SBA, Sheep blood agar; CAMP, Christie, Atkins, Munch-Petersen test; S/R, *Staphylococcus aureus/Rhodococcus equi; L.g., L. grayi;* BG, blue green.

Table 4. Antibiotic sensitivity pattern of *L. monocytogenes* isolates

Antibiotics	No. of Isolates (4)			Percentage		
	Resistant	Moderately sensitive	Sensitive	Resistant	Moderately sensitive	Sensitive
Amikacin	0	0	4	0.00%	0.00%	100.00%
Nalidixic acid	4	0	0	100.00%	0.00%	0.00%
Ampicillin	1	0	3	25.00%	0.00%	75.00%
Oxytetracycline	2	1	1	50.00%	25.00%	25.00%
Chloramphenicol	1	0	3	25.00%	0.00%	75.00%
Amoxicillin	1	0	3	25.00%	0.00%	75.00%
Cephotaxime	4	0	0	100.00%	0.00%	0.00%
Penicillin	4	0	0	100.00%	0.00%	0.00%
Erthromycin	1	1	2	25.00%	25.00%	50.00%
Ceftriaxone	1	1	2	25.00%	25.00%	50.00%
Kanamycin	4	0	0	100.00%	0.00%	0.00%
Gentamycin	0	0	4	0.00%	0.00%	100.00%
Norfloxacin	0	0	4	0.00%	0.00%	100.00%
Doxycyclin	0	0	4	0.00%	0.00%	100.00%

pathogenic species in the genus *Listeria* exhibit PI-PLC activity (Mengaud *et al.* 1991). Likewise, all *L. monocytogenes* isolates possessed *hly*A gene as well as displayed haemolysis on sheep blood agar (SBA) and CAMP test. The production of haemolysin is an important characteristic, which is directly related to the pathogenicity of *Listeria*, since non-haemolytic *Listeria* species can be considered as non-pathogenic (Courtieu 1991).

L. grayi isolate was non-haemolytic on sheep blood agar (SBA); CAMP test, and negative for PI-PLC assay. These were in perfect correlation with PCR where it lacked the amplification of any virulence genes.

The standardized PCR was assessed for its sensitivity to directly detect *L. monocytogenes* from milk. It was observed that PCR targeting *hly*A gene could detect 8.8×10^7 cells/ml to 8.8×10^5 cells/ml in all 3 artificially contaminated raw milk samples. Hence, the minimum detection limit from milk was 8.8×10^5 cells of *L. monocytogenes*/ml of milk (Fig.3). Our findings can be correlated with the findings of Rawool *et al.* (2007) who got the minimum detection limit to be 3×10^6 cells/ml.

However, application of PCR for the direct detection of pathogens present in foods is limited because of complex composition of the starting materials, which contain inhibitors for PCR amplification (Rossen *et al.* 1992, Bickley *et al.* 1996).

The overall antibiotic sensitivity pattern of *Listeria* isolates towards 14 antimicrobial agents tested is illustrated in Table 4. These findings are in concurrence with findings of several workers (Rodas-Suarez *et al.* 2006, Arslan and Ozdemir 2008, Morobe *et al.* 2009), who revealed sensitivity of *Listeria* species towards gentamicin as 76.4, 87.2, and 84.21%, respectively.

Serum samples (169) from cattle and buffaloes were screened for the detection of antibodies against LLO (ALLO) by using LLO-based indirect plate ELISA. After preliminary trials, antigen (LLO) at the concentration of 5,000 ng/ml, serum dilution at 1:100 and anti-species HRPO conjugate at 1:5,000 were standardized for final screening

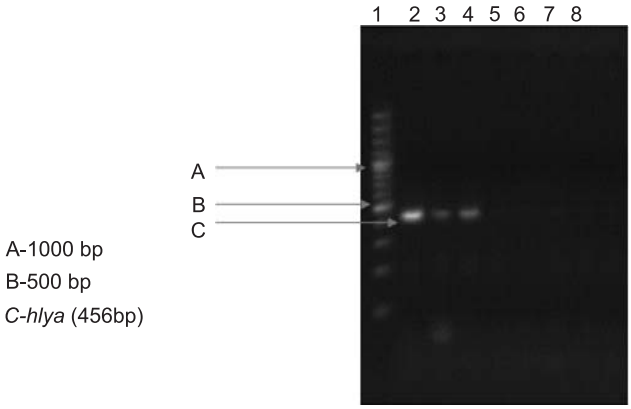

Fig. 3. Sensitivity of PCR for detection of *hlyA* in standard *Listeria monocytogenes*. Lane 1, PCR marker (100 to 3000 bp); Lane 2, 8.8×10^7 cells/ml; Lane 3, 8.8×10^6 cells/ml; Lane 4, 8.8×10^5 cells/ml; Lane 5, 8.8×10^4 cells/ml; Lane 6, 8.8×10^3 cells/ml; Lane 7, 8.8×10^2 cells/ml; Lane 8, 8.8×10 cells/ml.

of test sera. On screening of bovine sera, the seroprevalence turned out to be 7.1%.

Nigam *et al.* (1999) reported overall seroprevalence of 13.8% in cows and 14.7% in buffaloes in Himachal Pradesh; Kalorey *et al.* (2006) reported 5.48% seroprevalence from Vidarbha region of India, using listeriolysin-O as an antigen by indirect ELISA.

The detection of pathogenic, multi-drug resistant *L. monocytogenes* serotype 4b isolates with a prevalence of 1.14% from bovine milk and significant seroconvergent status (7.1%) among bovines in Punjab region of North India stands an important finding from public health point of view. PCR targeting *hly*A gene for direct detection of *L. monocytogenes* from milk could detect a minimum of 8.8×10^5 cells of *L. monocytogenes*/ml of milk.

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