PHENO-GENOTYPIC CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* FROM BOVINE CLINICAL MASTITIS

Mahendra Mohan Yadav, Ashish Roy, Bharat Bhanderi* and Chaitanya Joshi

ABSTRACT

The present study was carried out for pheno-genotypic characterization of *L. monocytogenes* (*L. monocytogenes*). A total of three isolates of *L. monocytogenes* were recovered from 85 mastitic milk samples (47 buffalos and 38 cows). Confirmation of the *L. monocytogenes* were based on biochemical tests followed by phenotypic characterization by hemolysis on sheep blood agar, the Christie Atkins Munch-Petersen (CAMP) test, phosphatidylinositol-specific phospholipase C (PIPLC) assay and phosphatidylcholine-specific phospholipase C (PIPLC) assay. The isolates were subjected to genotypic characterization with the help of PCR assay for five virulence associated genes namely, plcA, prfA, hlyA, actA and iap. The *L. monocytogenes* isolates were further subjected to multiplex-PCR based serotyping. All the three isolates of *L. monocytogenes* were hemolytic, CAMP positive, PI-PLC, PC-PLC positive, hlyA, pclA, actA, iap and prfA positive by PCR. All the three isolates of *L. monocytogenes* were serotyped as 4b.

Keywords: *Listeria monocytogenes*, clinical mastitis, PCR

INTRODUCTION

Mastitis is still a multi-etiological disease causing heavy economical losses to the dairy industry throughout the world. In India, the first report of mastitis by Dhanda and Sethi (1962) reported an annual economic loss of Rs. 529 million and thes increased to Rs. 60.5321 million annually in the year 2001 (Dua, 2001). Listeric mastitis, which is the most stubborn and difficult type to treat, results in culling of the infected animals from a herd (Stewart, 1998). It affects one or all the quarters, and the organism could be excreted for months, posing a potential threat to public health (Hird and Genigeorgis, 1990). Moreover, the naturally occurring cases of listeric mastitis cases may go unnoticed or undetected due to lack of suitable techniques employing specific media / antigen(s). The disease has been recognized as an emerging food-borne bacterial infection and public health peril. Outbreaks of food-borne listeriosis have been linked to dairy products, so attention has been focused in identifying animal reservoirs of listeriae in order to better understand the transmission of the disease (Farber and Peterkin, 1991). For both the sporadic and epidemic human listeriosis cases, ingestion of contaminated food is considered to be the primary source of infection (Schlech *et al.*, 1983). Since all major outbreaks of the invasive form of listeriosis are due to serovar 4b strains, an infrequent serovar in foods compared to 1/2a strains (Farber and Peterkin, 1991; Buchrieser *et al.*, 1993) and also major serovar responsible for ruminant listeriosis

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, AAU, Gujarat - 388 001, India *E-mail: bbbhanderi@yahoo.co.in

(Rocourt and Seelinger, 1985; Radostitis *et al.*, 1994), the procedure adopted for outbreak investigations relies upon serovar characterization to provide valuable information for rapid screening of groups of strains. Although 13 serovars are described for the species *L. monocytogenes*, at least 95 per cent of the strains isolated from foods and patients are of serovars 1/2a, 1/2b and 4b (Seeliger and Hohne, 1979; Tappero *et al.*, 1995; Graves *et al.*, 1999). The pathogenic potential of *Listeria* isolates can be assessed by in vitro pathogenicity tests (phenotypic characters) such as hemolytic activity, phosphatidylinositol specific phospholipases C (PI-PLC) assay (Notermans *et al.*, 1991a), phosphatidylcholine specific phospholipase C (PC-PLC) assay and in vivo methods namely, chick embryo (Notermans *et al.*, 1991b) and mouse inoculation tests (Menudier *et al.*, 1991).

Polymerase chain reaction (PCR) has a tremendous potential for the detection of animal pathogens, and therefore, it has attracted much interest in clinical veterinary microbiology in recent years. However, the results obtained have rarely been analyzed in the light of the pathogenic potential of the isolate(s) by in vitro or in vivo pathogenicity test(s) or the natural cases of the disease or the phenotypic detection / expressions of virulence-associated genes. The rapid and reliable diagnosis of listeriosis has been suggested to be ideally based on the detection of virulence markers (genotypic characters) of *Listeria* spp. by molecular techniques, and preferably, on the expression of their activities by in vitro assays (Notermans *et al.*, 1991a). The present investigation was undertaken with a view to study the phenotypic and genotypic characters of *L. monocytogenes* from clinical mastitic cases of cows and buffaloes in and around Anand city of Gujarat state.

MATERIALS AND METHODS

Bacteria - The strains of *L. monocytogenes* 4b (MTCC 1143), Staphylococcus aureus (MTCC 1144), Rhodococcus equi (MTCC 1135), Escherichia coli (MTCC 443) used in the study were obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The strains of Staphylococcus aureus (ATCC 25923), Str. agalactiae (NCIM 2401), Bacillus spp. (ATCC 6638), Ps. aeruginosa (ATCC 27853) were obtained from Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, India.

Samples - A total of 85 mastitic milk samples were collected aseptically from buffaloes (47) and cows (38) of Gujarat state, India. All the samples were quickly transported to the laboratory under chilled conditions and stored at 4°C till processed.

Isolation of *Listeria* - Isolation of listeriae from the milk samples of the animals were attempted as per the US Department of Agriculture (USDA) method described by McClain and Lee (1988) after making necessary modifications.

Briefly, samples were enriched by two-step enrichment in University of Vermont (UVM) broth-I and II. In UVM-I, incubation was carried out at 30°C for 24 h, while in UVM -II medium incubation was carried out at 30°C up to 7 days, with intermittent streaking after 24 h, 48 h and after 7 days of incubation, simultaneously onto Dominguez-Rodriguez isolation agar (DRIA), PALCAM agar, Oxford agar.

Confirmation of the isolates - Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20-25°C, methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and α-methyl-D-mannopyranoside).

Phenotypic characterization

Haemolysis on sheep blood agar (SBA) - All the *Listeria* isolates were tested for the type (α or β) and the degree (narrow or wider) of hemolysis on 7% sheep blood agar (SBA). Briefly, the isolates were streaked onto 7% SBA plates and incubated at 37°C in a humidified chamber for 24 h and examined for hemolytic zones around the colonies. Interpretation of the hemolytic reaction was based on the characteristic β-hemolysis in the form of wider and clear zone of hemolysis representing L. ivanovii while a narrow zone of α-hemolysis was the characteristic of *L. monocytogenes* or L. seeligeri.

Christie, Atkins, Munch and Petersen (CAMP) test - All the *Listeria* isolates were tested by CAMP test as per the method of Anonymous (1994) with some modifications. Briefly, the standard strains of Staphylococcus aureus and Rhodococcus equi were grown overnight on 7% SBA plates at 37°C and their colonies were again streaked onto freshly prepared 7% SBA plates in a manner such that the streaks were wide apart and parallel to each other. In between the parallel streaks of S. aureus and R. equi the *Listeria* isolates were streaked at 90°C angle and 3 mm apart before incubating them at 37°C for 24 h. The plates were examined for enhancement of the hemolytic zone from partial hemolysis to a wider zone of complete hemolysis, if any, between a *Listeria* strain and the S. aureus or R. equi strain owing to the synergistic effect of their hemolysins in case of a CAMP-positive reaction. The *Listeria* isolates with CAMP-positivity against S. aureus were characterized as *L. monocytogenes* and those with CAMP positivity against R. equi were characterized as L. ivanovii.

Phosphatidylinositol-specific phospholipase C (PI-PLC) assay - All the biochemically characterized *Listeria* isolates were assayed for PI-PLC activity as per the method of Leclercq (2004) with certain modifications. In brief, the *Listeria* isolates were grown overnight onto 7% SBA plates at 37°C. All *Listeria* isolates were streaked on L. mono differential agar (Hi Media Ltd, Mumbai, India) in order to assess PI-PLC activity. The inoculated plates were incubated at 37°C in a humidified chamber for 24 h. On L. mono differential agar, light blue colonies showing a halo formation around the inoculation site were considered positive for PI-PLC assay.

Phosphatidylcholine-specific phospholipase C (PC-PLC) Assay - The egg-yolk opacity test was done to examine the phosphatidylcholine-specific phospholipase C (PCPLC) activity of the isolates. Tryptic soy agar (Hi Media Ltd. Mumbai, India) plates were prepared with 2.5 per cent egg-yolk emulsion (Hi Media Ltd. Mumbai, India) and 2.5 per cent NaCl, pH 6.5-7. *Listeria* isolates were streaked onto the agar surfaces and incubated at 37°C for 36-72 h and observed for formation of opaque zones surrounding the growth (Coffey *et al.*, 1996).

Genotypic characterization

Polymerase chain reaction (PCR) based detection of multiple virulence-associated genes

The primers for the detection of hemolysin gene (hlyA), regulatory gene (prfA), phosphatidylinositol phospholipase C gene (plcA), actin gene (actA) and p60 gene (iap) of *L. monocytogenes* used in this study were synthesized by Sigma Aldrich, USA. The details of the primer sequences are shown in Table 1. The PCR was standardized for the detection of virulence-associated genes namely, plcA, prfA, hlyA, actA and iap of *L. monocytogenes* by following the methodologies described (Furrer *et al.*, 1991; Notermans *et al.*, 1991a; Paziak-Domanska *et al.*, 1999; Suarez and Vazquez-Boland, 2001) with suitable modifications. In brief, the standard strain of pathogenic *L. monocytogenes* (MTCC 1143) was grown overnight in brain heart infusion broth at

37°C. The culture (approximately 0.5 ml) was then centrifuged in a microcentrifuge (Sigma, USA) at 6000 xg for 10 minutes. The recovered pellet was resuspended in 100 µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 minutes and then snap chilled in crushed ice. The obtained lysate (5 µl) was used as a DNA template in PCR reaction mixture. The PCR was standardized for the detection of virulence associated genes of *L. monocytogenes* by optimizing the different conditions that affect the sensitivity and specificity of the reaction such as the concentrations of biologicals namely, MgCl2 (1.5-2.2 mM), primers (0.1-0.5 µM), Taq DNA polymerase (0.5-2.0 U), annealing temperatures (50-60°C) and number of cycles for amplification of the target gene. Based on optimization trials, the standardized PCR protocol for a 50 µl reaction mixture included 5.0 µl of 10x PCR buffer (100 mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM MgCl2 and 0.01% gelatin), 1 µl of 10 mM dNTP mix (a final concentration of 0.2 mM; Sigma, USA), 4 µl of 25 mM MgCl2 (a final concentration of 2 mM) and 10 µM of a primer set containing forward and reverse primers (a final concentration of 0.1 µM of each primer), 1 U of Taq DNA polymerase (Sigma, USA), 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The PCR tube (0.2 ml) containing the reaction mixture was tapped thoroughly with a finger and then flash spun in a micro centrifuge to settle the reactants at the bottom. The DNA amplification reaction was performed in a Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 minutes followed by 35 cycles each of 15 seconds denaturation at 95°C, 30 seconds annealing at 60°C and 1 minute and 30 seconds extension at 72°C, followed by a final extension of 10 minutes at 72°C and held at 4°C. All the five sets of primers for virulence-associated genes were amplified under similar PCR conditions and amplification cycles. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L), stained with ethidium bromide (0.5 µg/ml) and visualized by a UV transilluminator (UVP Gel Seq Software, England).

Specificity of the PCR - The specificity of the standardized PCR was tested by screening the standard strains of *L. monocytogenes*, *Listeria* species as well as some other commonly prevalent and cross reacting bacterial species with the primers used in this study. The DNA template preparation from the test organisms and other PCR conditions were similar to those described earlier.

Multiplex PCR based serotype detection of *Listeria monocytogenes* isolates

The multiplex PCR assay was standardized for the detection of three major serovars of *L. monocytogenes* namely 1/2a, 1/2b and 4b, following the methodology as described by Doumith *et al.* (2004) with suitable modifications. The primers for detection of *L. monocytogenes* 0737 gene (lmo0737), transcriptional regulator gene (ORF2819), secreted protein gene (ORF2110) and phosphoribosyl pyrophosphate synthetase gene (prs) in this study were synthesized from Sigma Aldrich. The details of the primer sequence are shown in Table 1.

The PCR was set for 50 µl reaction volume. Initially for the detection of *L. monocytogenes* serotype by PCR, conditions were optimized by using varying concentrations of molecular biologicals (Sigma Aldrich), gradient annealing temperature and number of cycles for amplification of target genes. Based on these trials, the reaction mixture for PCR was optimized as follows: 5.0 µl of 10X PCR buffer (Ammonium sulphate) (consisting of 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl2 and 0.01% gelatin), 1.5 µl dNTP mix (10 mM, with a

final concentration of 0.2 mM), 4 µl of 25 mM MgCl-2 (final concentration 2 mM) and 100 µM of forward and reverse primer of each set i.e. 1/2a, 1/2b and 4b (final concentration 0.1 µM each) and 10 µM of forward and reverse primer of each set of *Listeria* spp. (final concentration 0.1 µM each) 2 units of Taq DNA Polymerase, 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The PCR tube (0.2 ml) containing the reaction mixture was flash spun on a micro centrifuge (Remi, C 24) to get reactants at the bottom. The reaction was performed in Px2 Thermal cycler (Thermo electronic corporation, USA) with a pre-heated lid. The cycling conditions included an initial denaturation at 94°C for 5 minutes. followed by 35 cycles each of 30 seconds denaturation at 94°C, 1 minute. 15 seconds annealing at 54°C and 1 minute. 15 seconds extension at 72°C. This was followed by a final extension of 10 minutes. at 72°C and 30 minutes. held at 4°C. After the reaction, PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

Table 1. Primers for amplification of virulence associated genes and serotypes of *L. monocytogenes*.

Gene	Primer Sequence (5'-3')		Product size (bp)	Reference
plc A	Forward	CTGCTTGAGCGTTCATGTCTCATCCCCC	1484	Notermans *et al.* (1991a)
	Reverse	CATGGGTTTCACTCTCCTTCTAC		
prf A	Forward	CTGTTGGAGCTCTTCTTGGTGAAGCAATCG	1060	Notermans *et al.* (1991a)
	Reverse	AGCAACCTCGGTACCATATACTAACTC		
act A	Forward	CGCCGCGGAAATTAAAAAAAGA	839	Suarez and Vazquez Boland (2001)
	Reverse	ACGAAGGAACCGGGCTGCTAG		
hly A	Forward	GCAGTTGCAAGCGCTTGGAGTGAA	456	Paziak-Domanska *et al.* (1999)
	Reverse	GCAACGTATCCTCCAGAGTGATCG		
iap	Forward	ACAAGCTGCACCTGTTGCAG	131	Furrer *et al.* (1991)
	Reverse	TGACAGCGTGTGTAGTAGCA		
lmo0737 serovar1/2a	Forward	AGGGCTTCAAGGACTTACCC	691	Doumith *et al.* (2004)
	Reverse	ACGATTTCTGCTTGCCATTC		
ORF2819 serovar 1/2b and 4b	Forward	AGCAAAATGCCAAAACTCGT	471	
	Reverse	CATCACTAAAGCCTCCCATTG		
ORF2110 serovar 4b	Forward	AGTGGACAATTGATTGGTGAA	597	
	Reverse	CATCCATCCCTTACTTTGGAC		
prs serovar all *Listeria* pp.	Forward	GCTGAAGAGATTGCGAAAGAAG	370	
	Reverse	CAAAGAAACCTTGGATTTGCGG		

RESULTS

Isolation of *Listeria monocytogenes* - From 85 mastitis milk samples collected from cows and buffaloes, three were found positive for *Listeria* spp., all of which were *L. monocytogenes*. Among the isolates, two were from buffalo and one was from cow.

Phenotypic Characters - All the three isolates of *L. monocytogenes* showed the characteristic enhancement of hemolytic zone with S. aureus. All the three isolates of *L. monocytogenes* were found to be pathogenic by PI-PLC and PC-PLC.

Genotypic Characters - The standardized PCR allowed amplification of virulence associated genes of *L. monocytogenes* namely, plcA, prfA, actA, hlyA and iap to their respective base pairs, 1484 bp, 1060 bp, 839 bp, 456 bp and 131 bp PCR products, respectively, each represented by a single band in the corresponding region of the DNA marker ladder. Each of the primers was found to be specific to the target gene as it specifically amplified the PCR product of that gene, accordingly, all the five genes were detected in standard strains of *L. monocytogenes*, whereas, none of the genes was detected in the cultures of the other bacterial species cultures. The five virulence-associated genes were detected in all the three *L. monocytogenes* isolates

The multiplex PCR was standardized for detection of three major serotypes of *L. monocytogenes* viz., 1/2a, 1/2b and 4b by targeting various genes like lmo0737, ORF2819, ORF2110 and prs which were coding proteins like unknown protein, putative transcriptional regulator, putative secreted protein and putative phosphoribosyl pyrophosphate, respectively. All the three isolates showed amplification of three molecular size bands viz., 471 bp, 597 bp and 370 bp corresponding to their genes, ORF2819, ORF2110 and prs, respectively, while no amplification of lmo0737 gene. During the study, all the isolates were biochemically characterized as *Listeria*, all the three isolates amplified 370 bp products corresponding to gene prs, which served as internal amplification control. Employing the multiplex PCR assay serotyped, all the three *L. monocytogenes* revealed serotype 4b. In India, this the first report of isolation of *L. monocytogenes* serotype 4b from the bovine clinical mastitis.

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).

From 85 mastitis milk samples collected from cows and buffaloes, three were found positive for *Listeria* spp., all of which were *L. monocytogenes*. Pure cultures of *L. monocytogenes* were isolated from infected quarters. In India, isolation of *L. monocytogenes* from Holstein-Friesian cattle suffering from acute mastitis has been reported (Shome *et al.*, 2003) and in buffalo (Verma *et al.*, 2001), subclinical mastitis in cattle and buffalo (Rawool *et al.*, 2007). This is the first report on clinical mastitis caused by *L. monocytogenes* in Gujarat state, which is one of the leading dairy industry states in India. It is very well established that *L. monocytogenes* exists and multiplies as a saprophytic organism in the soil and on plants as well as in sewage and river water (Farber and Peterkin, 1991). Thus, it is obvious that a large source of *L. monocytogenes* exists in and

around milking cows and buffaloes. Cases of bovine clinical mastitis due to *L. monocytogenes* appear to be rare and the systematic literature on this subject was scanty.

With the exception of L. seeligeri being hemolytic but nonpathogenic, the pathogenic strains of *L. monocytogenes* are hemolytic. Hence, all the three *L. monocytogenes* isolates, which were hemolytic, could be considered as potentially pathogenic. All three isolates were found to be pathogenic in all the assays and possessed all the five virulence-associated genes. A number of factors are involved in manifestation of virulence of *L. monocytogenes* (Vazquez-Boland *et al.*, 2001). It has been demonstrated that the *L. monocytogenes* phospholipases are essential determinants of pathogenicity. Of these, the activity of virulence factor called PI-PLC and PCPLC is expressed only by pathogenic spp. of *Listeria* i.e., *L. monocytogenes* (Notermans *et al.*, 1991a) and L. ivanovii (Leimeister-Wachter *et al.*, 1991), and has been found to be a reliable marker for discrimination between pathogenic and nonpathogenic *Listeria* species (Notermans *et al.*, 1991a). The positivity of all the three isolates of *L. monocytogenes* in PI-PLC assay can be explained based on the common regulation of the hlyA gene and the plcA gene by the prfA encoded protein.

All the three isolates of *L. monocytogenes*, showed an opaque zone surrounding the growth. Similar Coffey *et al.* (1996) reported that *L. monocytogenes* produces an opaque zone surrounding the growth and Erdenlig *et al.* (2000) reported a zone of opacity on egg yolk agar around the growth of *L. monocytogenes* isolated from channel catfish. Thus our finding was supported by the earlier reports. One phenotype closely related with virulence of *L. monocytogenes* was egg yolk agar opacification, a reaction revealing lecithinase or phosphotidylcholine-phospholipase C (PC-PLC) activity. *L. monocytogenes* produces one or both of the two distinct types of reaction on egg yolk agar, either a faint halo or a dense zone of opacity surrounding the colony. The lipolytic activity of *L. monocytogenes* strains that produces a zone of opalescence around colonies on egg yolk or lecithovitellin agar is related to phopholipase C activity. PC-PLC was 29 kDa protein produced by all virulent strains of *L. monocytogenes*, whereas distinct lecithin degradation was not expressed by other *Listeria* spp. The presence of PC-PLC and another phospholipase enzyme (PIPLC) were required for virulence, although detection of one is sufficient for the identification of pathogenicity. As detection of virulence factors was useful to assist in the identification and differentiation of *Listeria* species, this report shows that lecithinase activity can conveniently be detected within 36 h on a relatively inexpensive medium.

The PCR employed in the present study turned out to be specific for the individual detection of five virulence-associated gene(s), namely plcA, prfA, actA, hlyA and iap, found in *L. monocytogenes* with respective sets of primers giving no cross-reactions with other bacteria. These findings are commensurate with the published work for detection of hlyA gene (Paziak-Domanska *et al.*, 1999), plcA and prfA genes (Notermans *et al.*, 1991a), iap gene (Furrer *et al.*, 1991) and actA gene (Suarez and Vazquez-Boland, 2001).

The multiplex PCR was standardized for detection of three major serotypes of *L. monocytogenes* viz., 1/2a, 1/2b and 4b by targetting various genes like Imo0737, ORF2819, ORF2110 and prs coding for proteins like unknown protein, putative transcriptional regulator, putative secreted protein and putative phosphoribosyl pyrophosphate, respectively. During the study, there was an absence of a 691 bp amplification product, corresponding to serotype 1/2a and serotype 1/2b was also not detected, since none of the isolates showed only amplification of 370 bp and 471 bp product. All the

three isolates showed amplification of three molecular size bands viz., 471 bp, 597 bp and 370 bp corresponding to their genes, ORF2819, ORF2110 and prs, respectively. This was in accordance with the results obtained for the aforementioned genes by Doumith *et al.* (2004) confirming that all the isolates to be from serotype 4b and this is the first report of isolation of *L. monocytogenes* serotype 4b from bovine clinical mastitis. The virulence of *Listeria* also depends upon serovar. Menudier *et al.* (1991) reported that serovar 4b was found to be more virulent as compared to other serovars (1/2a and 1/2c) and it helps to explain its association with listeriosis not only in animals but also in human beings. Furthermore, serotype 4b is the predominant serotype responsible for the animal listeriosis and *Listeria*-associated food-borne outbreaks, so it may be of immense importance to consider these three *L. monocytogenes* isolates for the further epidemiological investigation. A similar finding was also recorded by Yeh (2004), who observed 4b to be predominant serotype isolated from organic chicken carcasses.

In conclusion, recovery of potentially pathogenic *L. monocytogenes* from cow and buffalo mastitic milk samples signifies the zoonotic potential of listeriosis. Thus studies regarding epidemiological and zoonotic potential of this *L. monocytogenes* need special emphasis for improved diagnosis, control and surveillance measures in this part of the globe.

ACKNOWLEDGEMENT

We thank the Dean/Principal, College of Veterinary Science and Animal Husbandry, Anand, Gujarat, India for providing necessary facilities to carry out the research work.

REFERENCES

Annonymous, 1994. Bureau of Indian Standards: Microbiology - general guidance for the detection of *Listeria monocytogenes*. Committee Draft, CD, 11290.

Buchrieser, C., R. Brosch, B. Catimel and J. Rocourt. 1993. Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. *Can. J. Microbiol.,* **39:** 395-401.

Coffey, A., F.M. Rombouts and T. Abee. 1996. Influence of environmental parameters on phosphatidylcholine phospholipase C production in *Listeria monocytogenes*: a convenient method to differentiate *L. monocytogenes* from other *Listeria* species. *Appl. Environ. Microbiol.,* **62:** 1252-1256.

Dhanda, M.R. and M.S. Sethi. 1962. Investigation of mastitis in India. *ICAR Res.* Series No. 35. New Delhi, India.

Doumith, M., C. Buchrieser, P. Glaser, C. Jacquet and P. Martin. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.,* **42:** 3819-3822.

Dua, K. 2001. Incidence, etiology and estimated economic losses due to mastitis in Punjab and in India - An update. *Indian Dairyman,* **53:** 41-48.

Erdenlig, S., J.A. Ainsworth and F.W. Austin. 2000. Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. *J. Food Protect.,* **63:** 613-619.

Farber, J.M. and P.I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.,* **55:** 476-511.

Furrer, B., U. Candrian, C. Hoefelein and J. Luethy. 1991. Detection and identification of *Listeria monocytogenes* in cooked sausage products

and in milk by in-vitro amplification of haemolysin gene fragments. *J. Appl. Bacteriol.,* **70:** 372-379.

Graves, L.M., B. Swaminathan and S.B. Hunter. 1999. Subtyping *Listeria monocytogenes*, p. 251-297. *In* Ryser, E.T. and E.H. Marth (eds.) *Listeria, listeriosis and food safety.* Marcel. Dekker. Inc., New York, N.Y.

Hird, D.W. and C. Genigeorgis. 1990. Listeriosis in food animals: clinical signs and livestock as a potential source of direct (non-foodborne) infection for humans. *In* Miller, A.J., J.L. Smith, G.A. Somakuti (eds.) *Foodborne Listeriosis.* Elsevier Sci. Publ., Amsterdam, The Netherlands, 31p.

Leclercq, A. 2004. Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. *J. Microbiol. Meth.,* **57:** 251- 258.

Leimeister-Wachter, M., E. Domann and T. Chakraborty. 1991. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is coordinately expressed with listeriolysin in *Listeria monocytogenes*. *Mol. Microbiol.,* **5:** 361-366.

McClain, D. and W.H. Lee. 1988. Devepolment of USDA-FSIS method for isolation of *Listeria monocytogenes* from raw meat and poultry. *J. Assoc. Offic. Anal. Chem.,* **71:** 660-663.

Menudier, A., C. Bosiraud and J.A. Nicolas. 1991 Virulence of *Listeria monocytogenes* serovars and *Listeria* spp. in experimental infection of mice. *J. Food Protect.,* **54:** 917-921.

Notermans, S.H., J. Dufrenne, M. Leimeister-Wachter, E. Domann and T. Chakraborty. 1991a. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. *Appl. Environ. Microbiol.,* **57:** 2666-2670.

Notermans, S.H, J. Dufrenne, T. Chakraborty, S. Steinmeyer and G. Terplant. 1991b. The chick embryo test agrees with the mouse bioassay for assessment of the pathogenicity of *Listeria* species. *Lett. Appl. Microbiol.,* **13:** 161-164.

Paziak-Domanska, B., E. Bogulawska, M. Wiekowska-Szakiel, R. Kotlowski, B. Rozalska, M. Chmiela, J. Kur, W. Dabrowski and W. Rudnicka. 1999. Evaluation of the API test, phosphatidylinositol-specific phospholipase C activity and PCR method in identification of *Listeria monocytogenes* in meat foods. *FEMS Microbiol. Lett.,* **171:** 209-214.

Radostits, O.M., D.C. Blood and C.C. Gay. 1994. *Veterinary Medicine,* 8th ed. ELBS Bailliere Tindall. London.

Rawool, D.B., S.V.S. Malik, I. Shakuntala, A.M. Sahare and S.B. Barbuddhe. 2007. Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases. *Int. J. Food Microbiol.,* **113:** 201-207.

Rocourt, J. and H.P.R. Seelinger. 1985. Distribution des especesdu genre *Listeria. Zbl. Bakt. Hyg.,* **259:** 317-330.

Schlech, W.F., P.M. Lavigne, R.A. Bortolussi, A.C. Allen, E.V. Haldane, A.J. Wort, A.W. Hightower, S.E. Johnson, S.H. King, E.S. Nicholls and C.V. Broome. 1983. Epidemic listeriosis-evidence for transmission by food. *N. Engl. J. Med.,* **308:** 203-206.

Seeliger, H.P.R. and K. Hohne. 1979. Serotyping of *Listeria monocytogenes* and related species. *Methods Microbiol.,* **13:** 31-49.

Shakuntala, I., S.V.S. Malik, S.B. Barbuddhe and D.B. Rawool. 2006. Isolation of *Listeria monocytogenes* from buffaloes with reproductive disorders and its confirmation by

polymerase chain reaction. *Vet. Microbiol.,* **117:** 229-234.

Shome, B.R., I. Shakuntala, R. Shome and A. Kumar. 2003. Isolation of *Listeria monocytogenes* from mastitis case in Holstein-Friesian cattle. *Indian Association of Veterinary Microbiologists, Immunologists and Specialists in Infectious Diseases. Annual Conference,* Umiam, Barapani, 7 Feb. Abs. No. PP:05, 52p.

Stewart, J. E. 1998. *Listeria monocytogenes.* http:www.bact.wisc.edu/scienceEd/Listeriamonocytogenes.html.

Suarez, M. and J.A. Vazquez-Boland. 2001. The bacterial actin nucleter protein ActA is involved in epithelial cell invasion by *Listeria monocytogenes.* PUBMED [Accession No. AF103807].

Tappero, J.W., A. Schuchat, K.A Deaver, L. Mascola and J.D. Wenger. 1995. Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts. *JAMA-J. Am. Med. Assn.,* **273:** 1118-1122.

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Domi′Nguez-Bernal, W. Goebel, B. Gonza′Lez-Zorn, J. Wehland and J. Kreft. 2001. *Listeria* Pathogenesis and Molecular Virulence Determinants. *Cl. Microbiol. Rev.,* **14:** 584-640.

Verma, S., R.C. Katoch, R. Chahota and A.K Mahajan. 2001. *Listeria monocytogenes* - A cause of mastitis in an Indian buffalo (*Bubalus bubalis*). *Buffalo Journal,* **3:** 425-427.

Yeh, E.T. 2004. *Characterization of Listeria monocytogenes isolated from retail organic chicken.* M. S. Thesis, University of Maryland, U.S.

**Continued from page 28*

Quinn, P., C. Barros and D.G. Whittingham. 1982. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.,* **66:** 161-168.

Surani, M.A.H., S.C. Barton and M.H. Kaufman. 1977. Development to team of chimeras between diploid parthenogenetic and fertilized embryos. *Nature,* **270:** 601-603.