Standardization and Evaluation of Listeriolysin 'O' based ELISA for the Serodiagnosis of Listeriosis in cattle and sheep

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CERTIFICATE

This is to certify that the thesis *entitled "**Standardization and Evaluation of Listeriolysin 'O' based ELISA for the Serodiagnosis of Listeriosis in cattle and sheep"*** submitted by **Mrs. SHWETHA. S, I.D. No. MVHK - 917** in partial fulfillment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE** in **VETERINARY MICROBIOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Place: Bangalore
Date: 24th May, 2013

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Affectionately Dedicated

To My

Beloved Parents and Guide

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LIST OF ABBREVIATIONS

actA	Actin polymerization protein
ALLO	Anti-listeriolysin O antibodies
ALOA	Agar *Listeria* according to Ottaviani and Agosti
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
cELISA	competitive Enzyme Linked Immunosorbent Assay
Cm	Centimeter
DEAE-C	DiEthyl Amino Ethyl Cellulose
^{o}C	Degree Celsius
DNA	Deoxyribonucleic acid
CDC	Centre for Disease Control and Prevention
cDNA	complementary De-oxyribo nucleic acid
dNTP	2'-deoxyribonucleocide-5 triphosphate
EB	Elution Buffer
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
et al.,	*et alia*
etc.,	*et cetera*
Fig.	Figure
gm(s)	Gram(s)
G+C	Guanine and Cytosine base content
G	Centrifugal force equal to gravitational force
hlyA	Hemolysin *A*
Hr	Hour
HRPO	Horse Radish Peroxidase
Iap	Invasion associated protein
iELISA	indirect Enzyme Linked Immunosorbent Assay
IPTG	Isopropyl-β-D-Thio-Galactopyranoside
IU	International Unit

Kb	Kilo base
kDa	Kilo Daltons
LLO	Listeriolysin O
LPM	Lithium chloride phenylethanol moxalactam agar
µ	Micron
µg	Microgram
µl	Microlitre
mA	milli Ampere
Min	Minute
NASBA	Nucleic acid based sequence amplification
NFW	Nuclease Free Water
OD	Optical Density
ODD	Ortho Dianisidine Dihydrochloride
OIE	*Office International des Epizooties*
OMPs	Outer membrane proteins
OPD	Ortho Phenylenediamine Dihydrochloride
%	Per cent
PAGE	Poly Acrylamide Gel Electrophoresis
PALCAM agar	Polymyxin-Acriflavin-Lithium chloride-Ceftazidime- Aesculin Mannitol Agar
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p. i	Post infection
Pmol	Picomole
plcA	Phosphatidylinositol phospholipase C
PMSF	Phenyl Methyl Sulphonyl Flouride
Psi	Pounds per square inch
RE	Restriction Enzyme
R-LPS	Rough Lipopolysaccharide
Rpm	Revolution per minute
rRNA	Ribosomal Ribonucleic Acid

RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
Sec	Seconds
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	N, N, N′, N′–Tetra Methyl Ethylene Diamine
U	Unit
USDA	United States Department of Agriculture
UVM 1	University of Vermont medium 1
UVM 2	University of Vermont medium 2
UV	Ultra Violet

INTRODUCTION

I. INTRODUCTION

Listeria monocytogenes an intracellular bacterial pathogen, widespread in nature and associated with listeriosis in both humans and animals. Although known to occur commonly in silage fed ruminants since decades and first identified as an animal disease, its recognition in 1980s as food borne pathogen in humans promoted intense research activities. The associated potential for significant contamination is exacerbated by virtue of the organism's ability to tolerate high salt concentrations and the capacity to multiply at refrigeration temperatures. Among six species in the genus Listeria; *Listeria monocytogenes* (*L. monocytogenes*) and *Listeria ivanovii* (*L. ivanovii*) are pathogenic. Infection with *L. ivanovii* (previously known as *L. monocytogenes* serotype 5) is limited to ruminants such as sheep and cattle (Low and Donachie, 1997 and Vazquez-Boland *et al*., 2001), whereas the other four Listerial species are saprophytes adapted for survival in soil and decaying vegetation. Among 12 recognized serovars of *L. monocytogenes* most of the clinical isolates belong to 1/2a and 4b.

The disease has worldwide distribution and reported more frequently in temperate countries, however in the Indian subcontinent; sporadic cases have been reported but occasionally as outbreaks (Malik *et al*., 2002). The majority of *L. monocytogenes* infections are subclinical, resulting in healthy asymptomatic animals that shed *L. monocytogenes* in their feces. Clinical listeriosis in livestock can occur either sporadically or as epidemics and mainly in the form of encephalitis, abortion, stillbirth and mastitis. Disease is somewhat seasonal; occurring more often in winter and early spring and it has been associated with silage feeding. In humans, disease is characterized by flu-like illness

to severe complications including meningitis, septicaemia, spontaneous abortion or listeriosis of the newborn and very high mortality rate of 30 per cent (Gasanov *et al*., 2005).

Infection by *L. monocytogenes* is mediated by a number of virulence factors, hemolysin (*hlyA*), phosphatidyl inositol phospholipase C (*plcA*), actin polymerization protein (*actA*) and invasion associated protein (*iap*). The most important of these is listeriolysin O (LLO), a pore-forming toxin encoded by the *hlyA* gene produced only in virulent strains of the species and its detection indicates the presence of pathogen.

There has been a growing attention to *L. monocytogenes* infections mainly due to rapidly increasing number of outbreaks in farm animals with significant economic losses due to abortions and new born mortalities. Sheep and goats usually have an acute form of listeriosis and death occurs in 4-48 hours and recovery is rare. Cattle, on the contrary, have a more chronic disease with survival for 4-14 days and potential spontaneous recovery with lasting brain damage. Despite an early diagnosis, Listeriosis in animals can rarely be cured with antibiotics, however the disease is generally fatal and therefore prevention is the key to listeriosis.

Further, *L. monocytogenes* is a zoonotic agent and decisive role in the prevention of food-borne listeriosis in humans is the reduction of the presence of *L. monocytogenes* in all the critical stages of the food production and the distribution chain, including the epidemiological surveillance of livestock. The Centre for Disease Control and Prevention (CDC) reported that, of all the foodborne pathogens tracked by the

CDC; *L. monocytogenes* had the second highest case fatality rate (21%) and the highest hospitalization rate (90.5%) (Hearty *et al.*, 2006).

Thus, sensitive and specific tests to identify *L. monocytogenes* infected animals are of great importance in carrying out epidemiological surveys to develop appropriate control strategies. The authentic diagnosis of listeriosis relies on isolation of the organism by selective enrichments and subsequent biochemical analysis, although the method is laborious and time consuming, classical cultivation techniques still remains "gold standard" with which other methods are compared. However, the use of serology for the study of listeriosis has been hampered in the past by rather poor performances of the available tests and even the conventional serological tests such as agglutination test employing crude listerial proteins are difficult to interpret owing to the cross reaction between *L. monocytogenes* antigens and other bacteria besides, failure to distinguish between antibodies developed against pathogenic *L. monocytogenes* from those developed against non-pathogenic (Chaudhari *et al*., 2004).

Research on the pathogenesis of listeriosis has identified many virulence factors specific for *L. monocytogenes* which could serve as antigens for new improved serological tests. The best known among them is listeriolysin 'O', a toxin involved in the intracellular spread of *L. monocytogenes* (Fsihi *et al*., 2001). Several studies in human medicine have demonstrated the potential of this toxin as an antigen for the serological diagnosis of listeriosis in humans (Berche *et al*., 1990) and even similar studies have shown that small ruminants (Lhopital *et al*., 1993, Low and Donachie 1991 and Low *et al*., 1992), calves orally infected with *L. monocytogenes* (Baetz and Wesley, 1995;

Barbuddhe *et al.*, 1998 and Barbuddhe *et al.*, 2000a) and dairy cows with intramammary infections also produce significant humoral responses toward listeriolysin O. Listeriolysin 'O'based ELISA screening not only helps in herd level diagnosis, but also play an important role in establishing epideomiological data to initiate control measures. Polymerase chain reaction assays targeting multiple key virulence factors and various other molecular methods involving monoclonal antibodies, DNA probes and PCR based technology have been developed of late which possess equal sensitivity and allow testing to be completed within 48 hr. More recently, molecular methods were developed that target RNA rather than DNA, such as RT-PCR, real time PCR or nucleic acid based sequence amplification. These tests not only provide a measure of cell viability but they can also be used for quantitative analysis. These new methods are currently used mainly in research but their considerable potential for routine testing in the future cannot be overlooked.

Serological tests employing purified LLO such as ELISA can still be considered a useful tool for a quick and economical processing of large number of samples. Keeping this in view, the current study was undertaken to standardize Listeriolysin O based ELISA for the serodiagnosis of Listeriosis in cattle and sheep by considering the following objectives.

1. Standardisation of Listeriolysin' O' based ELISA for the serodiagnosis of Listeriosis in cattle and sheep.
2. Evaluation of Listeriolysin' O' based ELISA with PCR and isolation.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Listeriosis is an important food borne illness of zoonotic importance. In most occasions the disease goes undiagnosed for want of reliable methods. For the purpose of meaningful understanding of the current study on the standardization and evaluation of an indirect ELISA for the diagnosis of Listeriosis in animals, the available relevant literature is reviewed here under the following sub headings.

2.1 Isolation

In 1911 Hulphers was the first to describe bacteria, *Bacillus hepatis* isolated from a colony of rabbits (McLauchlin, 2004 and Hulphers, 2004). The official discovery of *Listeria* dates back to 1926, when Murray and co-workers isolated *L. monocytogenes* as the etiological agent of a septicemic disease affecting rabbits and guinea pigs in their laboratory at Cambridge in England. Later in 1927, Pirie isolated similar bacteria from gerbils in South Africa and named the disease 'Tiger river disease' and bacteria *Listerella hepatolytica* after the name of a British surgeon, Lord Joseph Lister. In 1940, the bacterium was finally named as *Listeria monocytogenes*.

Mahajan, (1936) was the first to describe the Listeriosis in India, in a sheep from Hyderabad but the pathogen was first isolated by Vishwanathan and Iyyar in 1950 from an infected sheep in Madras.

Sharma *et al.* (1996) revealed the presence of *L. monocytogenes* and *L. ivanovii* in vaginal swabs of 331 goat and 216 migratory sheep flocks with a history of abortions, in Himachal Pradesh.

Vishwanathan and Uppal (1981) isolated *L.monocytogenes* 4b from stomach contents of one of 22 aborted fetuses in 111 abortions out of 800 lambings in Jammu and Kashmir.

Fedio *et al*. (1990) examined samples (milk samples, including bulk milk, individual cow samples and quarter samples and even environmental samples such as feed, water and feces) from the milking herd consisting of 72 animals housed in a free stall barn for the presence of *L. monocytogenes* using the selective enrichment methods and found that only one animal was infected with subclinical mastitis caused by *L. monocytogenes* serotype 1 and *L. monocytogenes* was isolated from one of nine samples of feces in the free-stall barn and a sample of pelletized sugar beet pulp.

Takashi *et al*. (1991) reported that prevalence of *L. monocytogenes* was 1.6 per cent in ileo-cecal contents of cattle (312) in Tokyo by using combination of Oxford- LPM media for isolation.

Jensen *et al*. (1996) in their 23-year period study on prevalence of *L. monocytogenes* in 1,132,958 in raw milk samples of cow from Denmark found out that the percentage of cows infected with *L. monocytogenes* varied from 0.01 to 0.1 per cent (mean 0.04%) and of herds with an infected cow from 0.2 to 4.2 per cent (mean 1.2%), showing a low but constant level of infection. This study showed that a low but constant percentage of Danish dairy herds have cows infected with *L. monocytogenes*.

Bhilegaonkar *et al*. (1997) examined a total of 121 milk samples comprising 50 individual cow milk of an organised dairy farm, 16 individual cow milk of a private dairy

farm, 35 pasteurized bulk tank milk and 20 non-pasteurized bulk tank milk by detailed bacteriological examination for the isolation of *L. monocytogenes*. Out of 16 Listeria isolates recovered. Seven were of *L. monocytogenes* (organised dairy farm-2, private farm-4 and non-pasteurized bulk tank milk-1) and remaining nine isolates belonged to other Listeria *spp*. (organised dairy farm-3, private dairy farm-4 and non-pasteurized bulk tank milk-2).

Gaya *et al.* (1998) analysed raw milk samples from bulk tanks of 114 farms in central Spain for presence of *Listeria* species over a period of one year and found that *L. monocytogenes* and *L. innocua* were detected in 3.62 and 2.71 per cent of 774 milk samples, respectively and reported that most farms (85.1%) produced milk apparently free from *L.monocytogenes* throughout the sampling period.

Thakur (2000) reported that 4.68 and 8.69 per cent of vaginal samples collected from cases of repeat breeding and aborted cows yielded pathogenic *L. ivanovii* respectively. However, pathogenic *L. monocytogenes* could not be isolated.

Menzies *et al.* (2000) made clinical and laboratory studies from 264 cases of toxic mastitis in cows in Northern Ireland and found that half (132) of the cases were solely due to *E. coli* infection and further 15 organisms which were isolated in pure culture contributed to 17 per cent of all the cases. *Staphylococcus aureus, Streptococcus dysgalactiae* and *Actinomyces* species (3% each) were the predominant organisms isolated in pure culture. Forty one (16%) of the samples yielded various combinations of two bacterial species. However, *L.monocytogenes* could not be isolated.

Chaudhari *et al*. (2001) attempted isolation of pathogenic *Listeria spp.* in bacteriological samples of buffaloes, by selective enrichment in University of Vermont Medium and plating onto Dominguez-Rodriguez isolation agar. *Listeria* spp. and *L. monocytogenes* were isolated from 8.8 and 2.4 per cent and 4.8 and 1.6 per cent of 125 each meat and blood samples, respectively.

Carlos *et al.* (2001) attempted bacteriological isolation for *Listeria* species from a total of 1300 raw milk samples obtained from 20 l bulk tanks at four different dairy farms in southeast of Mexico City. The samples were enriched for 48 hr at 30°C and plated onto McBride's Modified Agar (MMA) and suspect colonies were biochemically tested. Overall, 23 per cent of all raw milk samples examined tested positive for *Listeria* species; 13 per cent were positive for *L. monocytogenes*; six per cent for *L. ivanovii*; four per cent for *L. seeligeri* and one per cent for *L. innocua.*

Barbuddhe *et al.* (2002) isolated *L. monocytogenes* in meat and milk samples of buffaloes by selective enrichment in UVM and plating onto Dominguez-Rodriguez isolation agar of which 2.4 per cent of meat samples and 6.25 per cent of milk samples were positive for *L. monocytogenes.*

Chaudhari *et al.* (2004) isolated pathogenic Listeria spp. from bacteriological samples of buffaloes in Bareilly, by selective enrichment in University of Vermont Medium (UVM) and plating onto Dominguez-Rodriguez isolation agar and found that isolation rate of *L. monocytogenes* was 2.4 and 1.6 per cent of 125 each meat and blood samples, respectively and that of 125 each of feacal, nasal and vaginal swabs was 4, 2.4 and 2.4 per cent respectively.

Shakuntala *et al.* (2006) isolated *L. monocytogenes* from clinical samples (Vaginal, nasal and fecal swabs and blood of buffaloes with various reproductive disorders by selective enrichment in UVM and plating onto Dominguez-Rodriguez isolation agar. The occurrence was 4.4 per cent in repeat breeding buffaloes, 6.6 per cent in aborted and 6.25 per cent in endometritis cases.

Bhanu Rekha *et al.* (2006) isolated *L. monocytogenes* from 7.08 and 1.77 per cent of meat (113) and blood (113) samples of goat, respectively processed as per the USDA method.

Kalorey *et al.* (2006) isolated pathogenic *Listeria spp.* in faecal samples of captive wild animals in Nagpur, Hyderabad and Pune, India. *L.monocytogenes* was isolated from eight (16%) of 50 faecal samples from six different mammals and one bird.

Kumar *et al.* (2007) presented first report of listeriosis outbreak in the migratory flocks of sheep in Punjab, India and reported that 69 sheep were affected out of 930 animals (875 sheep and 55 goats) caused by *L. monocytogenes*, with cumulative morbidity, mortality and case fatality rate of 7.89, 7.08 and 89.85 per cent, respectively by pathological and isolation methods.

Kalorey *et al*. (2008) examined milk samples collected from 2060 dairy cows for the presence of *Listeria* species by enrichment and plating on selective agar. Listeria *spp*. were isolated from 139 (6.75%) samples, of which 105 (5.1%) were positive for *L. monocytogenes*; two (0.1%) for *L. seeligeri*, 18 (0.9 %) for *L. innocua* and two (0.1%) for *L. welshimeri*. Of 105 *L. monocytogenes* isolates 90 were positive for *hly*A gene and also

concluded that insufficient hygiene during milking, storage and transport of milk was probable sources of contamination with Listeria *spp*. in raw milk.

Simranpreet Kaur *et al.* (2010) processed a total of 333 fecal, genital swab and blood samples from 111 animals with a history of reproductive disorders (cattle, buffaloes, sheep and goats) of which 19.8 per cent were *Listeria* species and out of which two were *L. ivanovii* and 29 *L. monocytogenes* from various sources.

Atil *et al*. (2011) isolated a total of 46 *Listeria* spp. from 719 samples (milk, bulk tank swabs, cheese, feed, water, faeces and the environment) collected from 415 cattle and 304 sheep in Turkey and 17.4 per cent of isolates were identified as *L. monocytogenes* by conventional and PCR techniques.

Mathakiya *et al*. (2011) made phenotypic characterization of 28 *L. monocytogenes* field isolates obtained from different animal species by CAMP test and found that all 28 field isolates revealed positive reaction, of which twenty three isolates showed characteristics enhancement of haemolytic zone with *S. aureus* on five per cent sheep blood agar and five isolates showed weak haemolytic zone.

Rajeev Kumar (2011) studied implication of *Listeria* species in various food borne disease and reported that almost all cases (about 98%) of human listeriosis and 85 per cent of animal cases were due *L. monocytogenes* and also found that healthy cows could serve as reservoirs for *L. monocytogenes* by secreting the organism in milk. Whereas, *L. ivanovii* was frequently associated with abortions in sheep and in cattle though *L. seeligeri* and *L. ivanovii* were rarely implicated.

Ali *et al*. (2012) undertook the study to explore the occurrence of *L. monocytogenes* in gallbladder of cattle and sheep. Out of three hundred gallbladder sampled randomly from sheep and cattle, eight (2.7%) Listeria *spp* were recovered in six (4%) samples of sheep and two (1.3%) samples of cattle by using International Dairy Federation protocol and further all the isolates were confirmed as *L. monocytogenes* by API- Listeria system and the presence of haemolysin (*hly*A) gene.

2.2 Serology

Miettinen *et al.* (1990) studied antibody response in five goats experimentally infected with listeriosis by oral inoculation of *L. monocytogenes* using an enzyme immunoassay and recorded a marked and enhanced IgG antibody response in two youngest and most seriously ill test animals. In these, the elimination of *L. monocytogenes* from the gastrointestinal tract occurred simultaneously with the development of the highest antibody levels at 14 to 15 days postinoculation. Whereas, oldest test animal, showed no clinical symptoms and the shortest fecal carriage associated with a preexisting, persistent IgG antibody response. Two animals remained practically seronegative and an IgM antibody response was not recorded for any of the animals. The findings suggested that an association exists between the humoral immune defense against Listeria infections, the clinical course of the infection and the elimination of the Listeria organisms from the gastrointestinal tract.

Barbuddhe *et al.* (2002) reported that 2.4 and 10.1 per cent of meat samples and 6.25 and 26.1 per cent of milk were positive for *L. monocytogenes* and other *Listeria sp*.,

respectively by isolation and 25.35 per cent of serum samples were found to be seropositive tested by listeriolysin O (LLO)-based indirect ELISA.

Boerlin *et al.* (2003) found that 11 per cent of the healthy dairy cows in Switzerland simultaneously presented antibodies toward listeriolysin O and internalin A, and 48 per cent of the farms had one or several animals simultaneously positive as detected by recombinant listeriolysin O and internalin A antigens based ELISA.

Zundel *et al*. (2007) studied dose-effect by comparing listerial infection following single or repeated doses and the contamination of the environment with the excreted bacteria, by inoculating ewes orally with either 10^4, 10^6 or 10^{10} cfu *L. monocytogenes* once, or daily for 10 days. Serological responses were monitored with indirect ELISAs using recombinant listeriolysin O (LLO), internalin A (InlA) and internalin A-related protein (IrpA) and found that repeated low daily doses did not facilitate seroconversion, even when animals excreted *L. monocytogenes.* (10^6 cfu) when compared increased antibody response in single high dose (10^{10} cfu) of *L. monocytogenes* with predominant serological responses against purified and synthetic LLO antigens and IrpA antigens.

Kalorey *et al.* (2007) standardized Enzyme linked immunosorbent assays [indirect and avidin-biotin (A-B)] for detecting *L. monocytogenes* antibodies in bovine milk samples (n = 2060) and were further evaluated by comparison with bacteriological examination. *L. monocytogenes* was isolated from 105 (5.1%) milk samples collected from 52 farms. Anti-LLO IgG antibodies were detected from 137 and 112 milk samples when tested by indirect and A-B ELISA respectively. Of the 52 farms screened, 28 (53.8%) yielded one or more isolates of *L. monocytogenes* and 33 (63.5%) of the farms

had one or more animals simultaneously positive by one or both the assays for anti-LLO antibodies.

Rathod *et al*. (2008) screened 267 serum samples from buffaloes in Gujarat for the presence of listerial antibodies and recorded that seroprevalence of *L. monocytogenes* was 23.9 per cent by indirect ELISA.

Esteban *et al.* (2009) screened pooled fecal samples from Northern Spain by an automated enzyme-linked fluorescent immunoassay to estimate the prevalence of positive herds. Further positive samples were sub-cultured onto the selective and differential agar, Agar Listeria Ottavani & Agosti (ALOA) and isolated *L. monocytogenes* from 46.3 per cent of dairy cattle, 30.6 per cent beef cattle and 14.2 per cent sheep flocks and found 1.5 per cent of faecal shedders in sheep and 21.3 per cent in cattle.

2.2.1 Extraction and Purification of Listeriolysin-O and Immunoblotting

Geoffroy *et al*. (1987) purified extracellular hemolysin of *L. monocytogenes* from the bacterial culture grown in resin (Chelex) treated medium by thiol-disulfide exchange affinity chromatography. The protein was characterized and termed as listeriolysin 'O', a sulflydryl-activated toxin, sharing the classical properties with other bacterial sulfhydryl-activated toxins such as inhibition by very low amounts of cholesterol, activation by reducing agents and suppression of the lytic activity by oxidation and antigenic cross-reactivity with streptolysin O.

Low *et al*. (1992) purified LLO 58,000-Da protein from the supernatant fluid of a dialysis sac culture of *L. monocytogenes* by Sulfopropyl (SP)-cation-exchange

chromatography and used as purified antigen for the indirect ELISA. Further purified LLO was confirmed in immunoblots, using both convalescent antiserum and hyperimmune anti-LLO rabbit serum.

Lhopital *et al.* (1993) obtained a highly purified LLO by preparative isoelectric focusing. Wherein, overnight culture supernatant of *L. monocytogenes* adjusted to pH 6.0, was passed twice through a DEAE column and effluent containing LLO was concentrated approximately to 10 mL on a YM 10 membrane and finally purified by isoelectric focusing. Harvested fractions were analyzed by SDS-PAGE followed by Western blot analysis by using rabbit anti-LLO polyclonal antibody.

Baetz and Wesley (1995) purified LLO from the cell culture supernatant of *L. monocytogenes* grown in 4.8 liters of an iron-depleted trypticase-soy broth containing 0.6 per cent yeast extract at 42^{o}C for 16 h. The culture supernatant supplemented with glycerol (5% final concentration), acetic acid (50mM final concentration) and 2-mercaptoethanol (5 mM final concentration) and adjusted to the pH 4.95 with NaOH was adsorbed onto a Zetaprep SP 100 capsule and was eluted with a NaCl step gradient (0.2 M- 0.5 M). Further the LLO fractions, were purified by fast protein liquid chromatography on a Mono-S column and the purity of LLO was ascertained by silver staining a SDS-PAGE gel. The purified LLO was stored at -80^{o} C until used.

Traub and Bauer (1995) purified LLO of *L.monocytogenes* for application in ELISA by hydroxylapatite adsorption chromatography and Sepharose S ion exchange chromatography and found that LLO (58 kDa) obtained was pure in terms of SDS-PAGE and immunoblots with polyclonal rabbit immune sera.

Bourry *et al.* (1997) obtained purified LLO from *L. monocytogenes* 4b culture supernatant fluid by single step of adsorption chromatography and presence of LLO in the antigenic preparation was confirmed by immunoblotting with anti-LLO monoclonal antibody (MAb).

Barbuddhe *et al.* (2000a) extracted and purified LLO from the cell-free supernatant of an 18-hour-old bacterial growth in brain heart infusion (BHI) broth at 37°C, further supernatant was precipitated with 50 per cent saturated ammonium sulphate and the precipitate was dialysed overnight against PBS, pH 7.2 and was passed twice through diethylaminoethyl cellulose (DEAE-cellulose), column equilibrated with 0.005M Tris-HCl buffer, pH 6.0. The purity of the LLO as checked by SDS-PAGE gel revealed a homogeneous 58,000 kDa protein.

Shakuntala *et al.* (2006) purified LLO from the cell-free supernatant of an 18-hour-old bacterial growth in BHI broth at 37°C. The DEAE cellulose purified pooled fractions representing a single peak were analysed by SDS-PAGE, which revealed a homogeneous protein of 58 kDa representing LLO.

2.2.2 Enzyme Linked Immunosorbent Assay (ELISA)

Low and Donachie (1991) studied clinical and serum antibody responses in lambs to infection by *L. monocytogenes*. Serological responses to flagellin were examined by ELISA and to listeriolysin O by immunoblotting and observed that responses of the animals to flagellin were weak and inconsistent, but antibodies to listeriolysin O were detectable after both oral and subcutaneous challenge with IgG_1 response predominantly.

Berche *et al*. (1990) tested whether detection of antibodies against listeriolysin 'O' could be used to diagnose human listeriosis. They examined sera from 28 patients infected with *L. monocytogenes* and 101 controls by dot-blot titration with purified LLO and observed 27 patients (96.4%) with listeriosis produced specific anti-LLO. Anti-LLO was also detected in eight (15.6%) of 51 healthy controls and in six (12.0%) of 50 controls who had various bacterial, fungal and viral infections whereas anti-LLO titres did not exceed 100 in these two control groups suggesting that test might be useful for epidemiological surveys and for serodiagnosis of listeriosis, especially when bacteria cannot be isolated.

Baetz and Wesley (1995) developed dot-blot assay and an ELISA to detect listeriosis in dairy cattle (17) that detected anti-listeriolysin O antibodies in the serum of cows experimentally infected with *L. monocytogenes*. The increase in agglutinating antibody titer correlated very well early in the infection with the results of the ELISA and the dot-blot assays.

Gholizadeh *et al*. (1996) developed recombinant antigens for use in serological tests by expressing amino-terminal fragments of LLO 240 and 411 residues (fragments LLO240 and LLO411 respectively) in *E. coli* as fusion polypeptides with maltose-binding protein (MBP) and the purified fusion polypeptide MBP-LLO411 was evaluated as a diagnostic antigen in a Western blot assay. Twenty-one of 21(100%) serum samples obtained from patients with listeriosis and found to be positive for to anti-LLO antibody (ALLO) by a reference dot blot test reacted with MBP-LLO411, whereas 1 of 20 (5%) anti-streptolysin O antibody positive serum samples and 1 of 100 (1%) serum samples

from healthy adults were reactive and opined that, a polypeptide limited to 411 amino-terminal residues of LLO is a specific and sensitive antigen for the detection of ALLO.

Baetz *et al.* (1996) evaluated specificity of purified LLO as a antigen to detect humoral immune responses in sheep, orally infected with *L. monocytogenes, L. ivanovii* or *L. innocua* and observed that only the *L. monocytogenes* inoculated sheep had an elevated temperature (> 42^{o}C) and antibodies as assessed by an ELISA after 15 days post infection.

Barbuddhe *et al.* (2000a) observed serum antibody profiles against LLO during oral *L. monocytogenes* infection of calves (n=6) by indirect ELISA. Immunoglobulin G (IgG) antibodies to LLO were observed to appear as early as day eight post infection (p.i.) with persistent titers and a plateau registering only a slight decrease by day 126 p.i.

Barbuddhe *et al.* (2000b) compared the detection of anti-listeriolysin O (ALLO) antibodies in serum samples with isolation of *L. monocytogenes* from meat and milk samples of sheep and goats. Out of 201 samples (87 milk and 114 meat) tested, 17.64 per cent yielded Listerial species isolates. *Listeria monocytogenes* was isolated from 6.66 and 1.56 per cent of meat (60) and milk (64) samples of goats, respectively and 7.4 per cent of sheep meat (54) samples, whereas, seropositivity for ALLO was observed in 41.13 and 33.76 per cent of goats and sheep, respectively and concluded that culture positivity for *L. monocytogenes* and detection of ALLO did not show any agreement.

Chaudhari *et al.* (2001) compared indirect ELISA to detect antibodies against LLO and the recovery pattern of *L. monocytogenes* from bacteriological samples in the

buffaloe calves orally infected with pathogenic *L. monocytogenes* and observed that antibodies to LLO appeared by 7-10 days p.i., peaked between days 16 and 36 p.i. whereas, the pathogen was recovered at low rates as ALLO first appeared but was absent in the fecal, nasal and blood cultures as production of ALLO peaked.

Boerlin *et al.* (2003) used recombinant listeriolysin O and internalin A antigens in ELISA for the specific detection of anti *L. monocytogenes* antibodies in 1,652 cattle serum samples. The results showed sensitivities and specificities of 82 and 92 per cent, respectively, for the listeriolysin O ELISA, and 100 and 90 per cent respectively, for the internalin A ELISA.

Elezebeth *et al.* (2007) studied occurrence of antibodies against LLO in sera of those aborted, mastitic and apparently healthy goats. A total of 120 serum samples were tested by LLO based indirect ELISA of which 19.16 per cent turned out to be seropositive. The percentage of seropositivity was higher in aborted goats.

Shakuntala *et al.* (2006) used LLO purified by ion exchange chromatography in indirect ELISA for the detection of antibodies against LLO in serum samples of buffaloes with reproductive disorders. The optimal antigen concentration of 40 ng per well, serum dilution of 1:200 diluted in PBST and 1:2000 dilution of anti-bovine HRPO conjugate were used. Forty per cent of samples were positive by LLO based indirect ELISA out of 125 serum samples tested.

Bhanu Rekha *et al.* (2006) used LLO purified by ion-exchange chromatography for detection of ALLO antibodies by indirect ELISA in the goats experimentally infected

with pathogenic *L. monocytogenes*. Antibodies against LLO were detected in 21.68 per cent serum samples collected from the slaughtered goats.

2. 3 Detection of Listeria by PCR and Molecular methods

Hudson *et al*. (2001) aimed to develop a 24-hr system for the detection of *L. monocytogenes* by using a combination of immunomagnetic separation (IMS) of bacterial DNA extraction directly from food sample followed by its detection using multiplex PCR. The PCR method used a primer pair targeting the listeriolysin O gene of *L. monocytogenes* and the other pair for a region of 23S rRNA genes of Listeria, giving products of 706 and 239 bp, respectively. The combined IMS/PCR was found to be sensitive and gave appropriate results.

Medeiros and Farber (2001) developed one-step PCR assay for simultaneous identification and typing of *L. monocytogenes* by employing an arbitrary (RAPD) primer in combination with a species specific gene primer set. Primers for *hly*A, *prf*A, *isp* and *fl2*A and six RAPD primers were screened for their discriminatory abilities, which produced the gene amplicon of interest as well as discriminatory banding profiles form all strains analyzed.

Liu *et al*. (2003) designed PCR oligonucleotide primers targeting eight potential virulence genes (Putative internalin genes: *lmo*2821 and *lmo*2470; putative transcriptional regulator genes: *lmo*2672, *lmo*1134, *lmo*1116, *lmo*0834, *lmo*0833 and *lmo*1188) that were predominantly detected virulent *L. monocytogenes* isolates, as determined by a mouse virulence assay; and found that one of the putative internalin genes, *lmo*2821, was detected in all known virulent strains (19 of 29 strains tested)

indicating that these genes serve as markers for PCR assessment of *L. monocytogenes* virulence.

Amagliani *et al.* (2004) confirmed specificity of the primers LL7 and LL8 for *L.monocytogenes* by performing PCR amplification on DNA obtained from 33 isolates of *L.monocytogenes,* of which seven (21%) strains of Type 1, 25 (76%) of Type 2 and one (3%) of Type 3 (ATCC 9525 strain), as well as *L. innocua* (6 strains), *Staphylococcus aureus* (1 strain), *Salmonella abortus ovis* (1 strain), *Enterococcus faecalis* (2 strains), *Streptococcus β-haemoliticus* (1 strain) and *E. coli* henterotoxic HB LT+ (2 strains), which amplified a 172 bp region of the hlyA gene specific to *L. monocytogenes,* from all *L. monocytogenes* strains tested, but none from *L. innocua* strains and other bacterial species, including haemolysin-producing bacteria.

Balamurugan *et al*. (2004) standardized PCR by using set of primers targeting the *hly*A gene of *L. monocytogenes* which produced a 731 bp product and the amplicon was further confirmed by nested PCR and *Hinf* I digestion and found that the primers specifically amplified only *L. monocytogenes* isolates.

Amagliani *et al*. (2006) evaluated the usefulness of a combination of PCR and ELISA tests as a diagnostic mass-screening tool. The assays were carried out with two commercial kits detecting a species-specific DNA sequence the hlyA gene and anti-LLO IgG, respectively in symptomatic and asymptomatic sheep flocks. PCR amplified 172 bp gene fragment from milk DNA samples yielding positivity at the rate of 47 per cent for early phase and 8.7 per cent for later phase samples from symptomatic flock and none from asymptomatic flock. Whereas, ELISA detected that eight per cent of the samples

were positive at the first analysis while the percentage increased to 87 per cent after three months.

Liu *et al*. (2006) examined a collection of *L. monocytogenes* strains by PCR and Southern blot analysis using species, virulence, and serotype specific primers and probes, for the purpose of further investigating *L. monocytogenes* serotype 4b strains belonging to lineages I and III by using the recently reported species-specific *lmo* 073, virulence-specific *lmo* 2821. As assessed by PCR, the species-specific *lmo* 0733 primers recognized all 25 *L. monocytogenes* strains under investigation and the virulence-specific *lmo*2821 primers detected all *L. monocytogenes* strains except the serotype 4a strain and two serotype 4b lineage III strains. Southern blot analysis using species-specific *lmo* 0733 and virulence-specific *lmo* 2821 gene probes largely confirmed the PCR results.

Liu *et al*. (2007) developed multiplex PCR incorporating inlA, inlC and inlJ gene primers for rapid speciation and virulence determination of *L. monocytogenes*. The species identity of the 36 *L. monocytogenes* strains under investigation was verified through the amplification of an 800 bp fragment with the *inl*A primers and the virulence of these strains was ascertained by the formation of 517 bp and/or 238 bp fragments with the *inl*C and *inl*J primers respectively. Internalin gene *lmo*2821 (*inl*J) as examined individually in PCR, virulence specific *inl*J primers amplified 238 bp product from genomic DNA of 28 of the 36 *L. monocytogenes* strains and did not cross-reacted in PCR with the 15 Gram-positive and Gram-negative bacterial strains tested, nor with 22 non-monocytogenes Listeria strains and was identified as a useful target for rapid differentiation of *L. monocytogenes* virulent from avirulent strains.

Lelia *et al*. (2007) screened eight dairy farms in two distant geographic regions of Portugal, to evaluate the presence and distribution of Listeria spp. in their environment by an integrated analysis of RAPD fingerprints with three primers, molecular identification and genomic typing of isolates. Three dairy farms in each region were positive for the presence of listeriae and 213 isolates were obtained.

Rawool *et al*. (2007) subjected isolates of *L. monocytogenes* recovered from two fecal swab, one milk sample of three mastitic cattle and one buffaloe milk samples to virulence specific PCR assay targetting *plc*A, *prf*A, *hly*A, *act*A and *iap* genes and observed that all the five genes were detected in one of the isolate from the milk of mastitic cattle whereas, the remaining three isolates were found to possess *prf*A and *hly*A; *act*A, *hly*A and *iap*; *hly*A and *iap* respectively.

Ritu *et al.* (2008) compared isolation of *L. monocytogenes* with PCR targeting virulence associated genes (the *plc*A, *hly*A and *prf*A), all eight isolates from cattle milk samples were positive for *hly*A gene and *plc*A genes. However, six isolates of *L. monocytogenes* were positive for the *prf*A.

Frece *et al*. (2009) examined 180 milk products samples (domestic unpasteurised milk, fresh cheese and cream of raw milk) for *L. monocytogenes* using conventional (microbiological and biochemical - API test) and PCR methods. Of which 27.6 per cent were presumptively positive for Listeria on PALCAM agar and only 21.3 per cent of samples were confirmed to be positive for Listeria by API Listeria test whereas 17.3 per cent were confirmed to be positive for *L. monocytogenes* by PCR amplification of the *hly* gene (64 bp).

Singh *et al*. (2009) developed a real-time polymerase chain reaction duplex assay which has advantage of detecting *E. coli* O157:H7 and *L. monocytogenes* in tested milk and dairy food samples in a single reaction by using differently labeled molecular beacon probes targeting *rfb* gene of *E. coli* O157:H7 and the *hly* gene of *L. monocytogenes*, the duplex assay performed reasonably well in terms of sensitivity and specificity.

Mohammed *et al*. (2009a) reported that proportionsof *L. monocytogenes* in faecal samples from cows, calves and other animals in calf-cow operations in Central and Southerrn California was 3.1per cent, 3.75 per cent and 2.5 per cent respectively by using a combination of enrichment and polymerase chain reaction tests whereas, *L. monocytogenes* was less common in feed lot faecal (0.3%) and soil (0.75%) samples collected.

Mohammed *et al*. (2009b) tested various samples obtained from cattle (feces, composite udder milk, and udders), their environment (silage, feed bunks, water troughs, and floor bedding) in dairy cattle operations in central New York State by using PCR assay with two steps of bacterial enrichment and per cent prevalence of *L. monocytogenes* was 13, 19 and 43 in composite milk, udder swab samples and fecal samples at of respectively and also observed that the prevalence of *L monocytogenes* was twice as high in samples obtained from feed bunks, water troughs, and bedding, compared with that in samples obtained from silage (65%, 66%, 55% and 30% respectively), indicating that *L. monocytogenes* was more prevalent in samples obtained from dairy cattle and their environment than in milk samples. Strategies to control the

pathogen in dairy operations should focus on cow hygiene and sanitary milk harvesting on the farm.

Hye *et al*. (2009) compared individual genes in prfA virulence gene cluster (pVGC), *prf*A, *plc*A, *hly* A, *mpl*, *act* A and *plc* B in all *Listeria* species, primers for which yielded amplicons of 479 bp, 674 bp, 496 bp, 798 bp, 1994 bp and 320 bp respectively from all five strains of *L.monocytogenes* tested and none from other *Listeria* species and concluded that priming sites within genes are exclusively specific for *L.monocytogenes.*

Mahendra *et al*. (2010) tested three isolates of *L. monocytogenes* obtained from mastitis milk samples (85) of cows and buffaloes in Gujarat and found that all five virulence associated genes plcA, prfA, actA, hlyA and iap were amplified by all three isolates generating 1484 bp, 1060 bp, 839 bp, 456 bp and 131 bp PCR products, respectively.

Nayak *et al*. (2010) reported that 10 (6.7%) samples out of 150 buffalo meat samples were found positive for *Listeria* species, of which four (2.7%) were positive for *L.monocytogenes,* all 4 (2.7%) *L. monocytogenes* isolates identified by biochemical tests were subjected to PCR and all these isolates were successfully amplified the desired amplicon of 660 bp. The PCR was performed from each diluted culture and showed the amplification up to as low as 2x101 CFU/ml using primer pair of *iap* gene.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1 General considerations

The glassware used in this study were of neutral glass of Corning, Borosil India Ltd., or Scott Durham (Germany) make. The culture media, buffers and other biochemical reagents were prepared in quartz glass double distilled water. The chemicals of Analar, Excellar or molecular biology grade were used for the preparation of various solutions and reagents. The culture media, buffers, reagents, enzymes, kits and other requirements were obtained from M/s Bangalore GeNei, Bangalore; MWG Biotech AG, Bangalore; M/s. Hi-media, Mumbai; Sisco Research Laboratories Pvt. Ltd., Mumbai; Oxoid, England; Sigma Aldrich, USA; Fermentas Life Sciences, and Qiagen (Germany); EMerck (India) Ltd.

Plastic ware including micro centrifuge tubes, micropipette tips, cryovials, Petri dishes and autoclave bags were procured from M/s. Tarson Products Pvt. Ltd., Kolkata and Axygen Inc., USA.

3.1.1 Preparation of glassware

The glassware were prepared by soaking them in a detergent solution over night. Next day, they were washed thoroughly in running tap water followed by a rinse in de-ionized / distilled water. The air-dried glassware were packed and sterilized in hot air oven for one and half hours at 160^{o}C.

3.1.2 Preparation of plastic ware

The new plastic ware including micro centrifuge tubes and micropipette tips were sterilized by autoclaving at 121^{o}C for 15 min at 15 lb/Sq. inch.

3.2 Standard / reference strains

The standard strains of *Listeria monocytogenes* 4b (MTCC 1143) and *Listeria monocytogenes* 1/2a, maintained at PD_ADMAS, Bangalore were used in this study. These strains were tested for their purity, morphological and biochemical characteristics as per the protocol supplied by the manufacturer and further confirmed for their species using HiListeria^{TM} Identification Kit (Himedia Laboratories, Mumbai) and by species specific PCR targeting *Lmo 2821*. The stock culture was maintained by sub-culturing at monthly intervals in brain heart infusion (BHI) agar slants.

3.3 Clinical samples

A total of 325 and 302 clinical samples were collected from cattle and sheep respectively from different places of Karnataka by making visits and sera samples from seven other states were kindly made available by PD_ADMAS, Bangalore. The samples include serum (235 cattle and 252 sheep), Blood (40 each from cattle and sheep), vaginal swabs (10 each from cattle and sheep) and 40 milk samples from mastitis cows. Vaginal swabs were collected from animals with various reproductive disorders or history of abortion and apparently healthy animals. Distribution of samples collected from different species and states is set out in table 1. Sera samples were stored at -20^{o} C until tested. Other clinical samples (Milk, vaginal swabs and blood) were stored at 4^{o} C until processed for Listerial isolation.

Table 1. Screening of clinical samples from various states for Listeriosis by ELISA, PCR and isolation

Sl No.	State	Cattle		Sheep	
		Sera for ELISA	Samples for Isolation/ PCR**	Sera for ELISA	Samples for Isolation /PCR**
1	Karnataka	65	20* (blood) 40 (Mastitis Milk) 10 (Vaginal swabs)	80	20* (blood) 10 (Vaginal swabs)
2	Maharashtra	90	20* (blood)	33	20* (blood)
3	Rajasthan	40	-	14	-
4	Manipur	32	-	32	-
5	Gujarat	-	-	43	-
6	Andhra Pradesh	-	-	17	-
7	Orissa	-	-	33	-
8	Tamilnadu	8	-	-	-
	Total	**235**	**90 (40**)**	**252**	**50 (40**)**

* Samples common for PCR and isolation; respective sera were evaluated in ELISA,
** Samples processed for PCR

3.4 Isolation of *Listeria monocytogenes* from clinical samples

Isolation was attempted from 90 cattle samples (40 blood, 40 mastitis milk sample and 10 vaginal swabs) and 50 sheep samples (40 blood samples and 10 vaginal swabs) as per United States Department of Agriculture (USDA) method (Mc Clain and Lee, 1988) with some modifications, using University of Vermont medium 1 (UVM) and UVM 2, a two step enrichment process, followed by streaking onto PALCAM media. UVM (UVM formulations Oxoid, England), is a Listeria Selective Enrichment Media, made based on the original formulation described by Donnelly and Baigent (1986) for the isolation of Listeria.

3.4.1 Media for isolation of *Listeria monocytogenes*

UVM medium (Oxoid, England)

The media composed of Listeria enrichment broth base which was prepared, autoclaved and followed by a separate addition of primary and secondary Listeria selective enrichment supplements just before the use, to get UVM 1 and UVM 2 respectively.

i. Listeria enrichment broth base (UVM Formulation)

Contents of the Medium	**gm/litre**
Proteose peptone	5.0
Tryptone	5.0
Lab-Lemco powder	5.0
Yeast extract	5.0
Sodium chloride	20.0
Tryptone	5.0
Disodium hydrogen phosphate	12.0

Potassium dihydrogen phosphate	1.35
Aesculin	1.0

ii. Listeria primary selective enrichment supplement (UVM 1)

Vial contents	**mg / vial**	**mg / litre**
Nalidixic acid	10.0	20.0
Acriflavine hydrochloride	6.0	12.0

iii. Listeria secondary selective enrichment supplement (UVM II)

Vial contents	**mg / vial**	**mg / litre**
Nalidixic acid	10.0	20.0
Acriflavine hydrochloride	12.5	25.0

Listeria enrichment broth base was reconstituted by dissolving 27.2 g in 500 ml of sterile distilled water and sterilized by autoclaving at 121^{o} C, 15 lb pressure for 15 min. So prepared media will give a pH of 7.4 ± 0.2 at 25°C. One vial of Listeria Primary Selective Enrichment Supplement (UVM I) was rehydrated with two ml sterilized distilled water and added aseptically to 500 ml of the above base. Similarly UVM II was also prepared by addition of rehydrated Listeria secondary selective enrichment supplement into 500 ml of broth base. Broths so prepared were mixed well and dispensed into aliquots of 25 ml in sterile flasks and stored at 4^{o}C until further use.

3.4.2 PALCAM Agar medium

PALCAM medium, a selective and differential diagnostic medium for the isolation of *L. monocytogenes* used in this study was based on the formulation described by Van Netten *et al.* (1989).

3.4.2.1 PALCAM Agar Base

Contents	g/litre
Columbia Blood Agar Base	39.0
Yeast extract	3.0
Glucose	0.5
Aesculin	0.8
Ferric ammonium citrate	0.5
Mannitol	10.0
Phenol red	0.08
Lithium chloride	15.0

3.4.2.2 PALCAM selective supplement

Contents	mg/ litre
Polymyxin B	10.0
Acriflavine hydrochloride	5.0 mg
Ceftazidime	20.0 mg

Palcam agar base was prepared by suspending 34.5 gm of base in 500ml of distilled water, sterilized by autoclaving at 121°C for 15 min. Once the PALCAM base was cooled to 50°C, the contents of one vial of PALCAM selective supplement rehydrated in two ml of sterile distilled water was added aseptically, mixed well and poured into sterile Petri dishes.

3.5 Extraction and purification of Listeriolysin 'O' antigen:

Extraction and purification of listeriolysin 'O' antigen was carried out as per the method described by Shakuntala *et al*. (2006). Briefly, the procedure includes,

i. A loopful of *L.monocytogenes* (1/2a) from BHI agar slant was inoculated into 50 ml of BHI broth and incubated at 37^{o}C for overnight.

ii. Over night culture was transferred into 500 ml of BHI broth and incubated at RT for 12 hrs on an orbital shaker at a speed of 150 rpm.

iii. Culture was centrifuged at 14,000g at 15^{o}C for 15 min, supernatant was collected into a separate sterile flask.

iv. The supernatant was then processed for salting out with ammonium sulphate 50 per cent (175gm for 500ml volume of supernatant), further, 1mM of PMSF added just before salting and stirred for 3 hr on a magnetic stirrer at refrigeration temperature, then centrifuged at 14,000 g, at 15^{o}C for 15 min.

v. The pellet was collected into a minimum quantity of sterile PBS (pH 7.2) and supernatant was discarded.

vi. The dissolved pellet was then dialyzed at refrigeration temperature, twice against PBS (pH 7.2) on a magnetic stirrer for one and half hour each time.

vii. Final dialysis was carried out over night at refrigeration temperature without magnetic stirrer by replacing the PBS (pH 7.2) with Tris-HCl (pH 6) and 1mM PMSF added to dialyzed material.

3.5.1 Diethyl Amino Ethyl Cellulose (DEAE-C) column purification

Crude extract adjusted to pH 6 obtained in 7th step of the section **3.5** was loaded onto DEAE-C column for purification**.**

i. Pre-cycling of ion exchanger was carried out by stirring 10 g of DEAE cellulose (Whatman, DE 52) with 15 volumes of 0.1N NaOH, followed by the filtration of suspension after 30 min of saturation. Later the supernatant was washed with distilled water until an intermediate pH of 8 was obtained. Then the filtrate was stirred with 0.1N HCl and left for 30 min followed by filtration and washing with distilled water until filtrate is near neutral.

ii. Activated DEAE cellulose obtained in step 1 was filled into a column; care was taken to avoid any air bubbles. The column was then equilibrated with Tris HCl 0.05M (pH 6) until pH of eluent is 6.

iii. The dialysed material obtained in vii step of the section **3.5** was applied onto the column and proceeded for elution with tris HCl pH 6.

iv. The elutes were collected in fractions of 1.5 ml each into sterile tubes.

v. First three fractions were discarded, while rest other 30 fractions were stored at 4^{o}C.

vi. The fractions so obtained were subjected to spectrophotometer to take optical density (OD) at 280 nm.

vii. Fractions were pooled according to major peaks of absorbance at 280 nm and then concentrated by reverse dialysis using sucrose.

viii. Protein estimation was done by Bradford's dye binding method. (Ms Bangalore GeNei, Bangalore)

ix. Fractions were subjected to SDS-PAGE for characterization of protein and confirmed by western blot analysis.

3.6 Characterization of protein by SDS-PAGE

3.6.1 Equipments and Reagents

Equipments

SDS-PAGE apparatus (Bio-Rad)

Power pack (Bio-Rad)

Dry bath

Reagents

1. Sample buffer

Tris-HCl (0.5M, pH 6.8)	:	0.5 ml
Glycerol	:	0.4 ml
SDS (10%)	:	0.8 ml
β-mercaptoethanol	:	0.2 ml
Bromophenol blue (1% w/v)	:	0.2 ml

The volume made up to 4 ml with glass distilled water.

2. Separating gel (10%, 5 ml)

Distilled water	:	1.9 ml
Acrylamide - bisacrylamide (29:1)	:	1.7 ml
Tris- HCl (1.5 M; pH 8.8)	:	1.3 ml
SDS (10%)	:	50.0 µl
Ammonium per sulfate (10%)	:	50.0 µl
TEMED	:	10.0 µl

3. Stacking gel (4.5%, 3 ml)

Distilled water	:	1.5 ml
Acrylamide-bisacrylamide (29:1)	:	540.0 µl
Tris- HCl (0.5M, pH 6.8)	:	900.0 µl
SDS (10%)	:	30.0 µl
Ammonium per sulfate (10%)	:	30.0 µl
TEMED	:	3.0 µl

4. Tris glycine Running buffer (pH 8.3)

Tris base (Tris-25mM)	:	3.0 g
Glycine (Glycine-250mM)	:	14.4 g
SDS (SDS-0.1%)	:	1.0 g

The volume made upto 1L with Distilled water

5. Protein staining solution

Coomassie brilliant blue R-250	:	0.250 mg
Methanol	:	45.0 ml
Glacial acetic acid	:	10.0 ml
Distilled water	:	45.0 ml

6. Protein destaining solution

Methanol	:	45.0 ml
Glacial acetic acid	:	10.0 ml
Distilled water	:	45.0 ml

3.6.2 Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS – PAGE)

The Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis charecterization of the proteins was carried out as per the protocol described by Laemmli (1970). About 30 µl of each protein fraction aliquot was mixed with equal volume of 2X sample buffer and heated at 95°C on dry bath for five min. The samples were analyzed in 10 per cent (SDS – PAGE) gels using protein molecular weight markers to study the protein profile and estimate the molecular weight of the protein fraction of our interest.

A 30 per cent stock solution of acrylamide and bisacrylamide (29:1 w/w) was prepared and used for gel preparation. The stacking and separating gels of the required percentage were prepared. The gel casting platforms were assembled and the bottom of the assembly was sealed. The separating gel was poured in-between the glass plates placed two mm apart using spacers and layered with a thin layer of water saturated n-butanol as a barrier and gel was allowed to set. The over layered water saturated n-butanol was removed and the residual butanol was flushed out with distilled water and traces of water was blotted off before the stacking gel (4.5%) was layered above the separating gel and a comb placed immediately to create wells. The polymerized gel was transferred to an electrophoretic apparatus and the buffer chambers filled with tris glycine running buffer (pH 8.3). Protein samples prepared earlier were loaded into the wells after washing them with the running buffer. The electrophoresis was carried out at 100V till the dye reached the bottom of the separating gel.

3.6.3 Staining and detection of protein bands

After the completion of electrophoresis, gels were stained with Coomassie brilliant blue according to Sambrook and Russel (2001).

i. The gel was carefully separated from the glass mould, immersed in five volumes of staining solution and placed on a slowly rotating platform for six hrs at RT.

ii. The stain was removed and saved for future use.

iii. The gel was de-stained by soaking in the destainer solution on a slow rotating platform by changing the destaining solution several times until polypeptide bands were clearly visible.

iv. After destaining, the gels were stored in distilled water with 10 per cent glycerol.

v. A permanent recording of the stained gel was made by either photographing or documenting in gel documentation system (AlphaImager^{TM} 2200 from Alpha Innotech Corporation, USA).

vi. The molecular weight of the protein bands was determined in comparison with the standard protein molecular weight markers using software provided along with the gel documentation system.

3.7 Confirmation of protein by western blot analysis

3.7.1 Equipments

a. TransBlot–SD electrophoretic transfer cell (Bio-Rad)
b. Power pack (M/s Bangalore Genei, Bangalore)
c. Nitrocellulose membrane (Pall Corporation, USA)
d. Whatman No. 3 filter paper

Reagents

i. Transfer buffer

Tris HCl (pH 8.3)	:	3.0 gm
Glycine	:	1.44 gm
Methanol	:	20.0 ml

The volume was made up to 100 ml with Distilled water.

ii. PBS-T (Wash buffer)

Tween 20	:	100 µl
PBS	:	100 ml

The volume made up to 500 ml with Distilled water.

iii. Blocking Solution

Tris HCl, pH 7.5	:	0.1211 g
NaCl	:	0.8775 g
Skim milk powder	:	5.0 g
Tween 20	:	0.05 ml

The volume was made up to 100 ml with Distilled water.

iv. Acetate buffer (0.2M)

Solution A

Acetic acid	:	6.0 ml
Distilled water	:	500.0 ml

Solution B

Sodium acetate	:	16.4 g
Distilled water	:	1000.0 ml

148 ml of solution A and 352 ml of solution B gives 500 ml of Acetate buffer of pH .

v. Substrate Buffer stock for immune-detection (4X)

Acetate buffer (pH 5.0)	:	50.0 ml
EDTA	:	74. 4 mg
Triton X-100	:	0.4 ml

Stored at 4^{o}C.

vi. Substrate solution for immune-detection.

Substrate buffer (1X)	:	25.0 ml
O-Dianisidine Dihydrochloride (ODD)	:	5.0 mg
Hydrogen peroxide	:	16.0 µl

Protein extracts of cell free culture filtrate, that were characterized by SDS-PAGE, were further subjected to Western blot analysis as per the method described by Sambrook and Russel (2001). After running the SDS-PAGE, the protein bands from the gel were transferred on to a nitrocellulose membrane using TransBlot–SD electrophoretic transfer cell (Bio-Rad) according to manufacturer's instructions.

3.7.2 Electro-blotting

i. The SDS-PAGE electrophoresed gel was washed in transfer buffer for three times at five min intervals.

ii. The nitrocellulose membrane was cut to the exact size of the gel, pre-wetted with transfer buffer for five min, gently agitated, washed with distilled water and equilibrated in transfer buffer for five min.

iii. Three sheets of Whatman No. 3 filter paper, cut to the exact size of the gel, were soaked in the transfer buffer. These soaked filter papers were placed on the flat anode plate over which the pre-wetted NC membrane was placed and pressed gently to exclude excess of buffer and air bubbles

iv. The gel was placed carefully over the membrane, its orientation was marked, transfer buffer was poured over the gel and pressed carefully to exclude excess buffer and air bubbles.

v. Three sheets of Whatman No. 3 filter papers, soaked in transfer buffer, were placed over the gel and excess buffer and air bubbles were squeezed out.

vi. Cathode plate was placed in position and transfer of proteins was done at 15V for 35 min.

vii. At the end of transfer, the NC membrane was separated out and the gel was stained as done earlier section **3. 6. 1** to check for the efficiency of protein transfer.

3.7.3 ELISA

The electro transferred membrane was subjected for Western blot analysis with listeria convalescent sera from cattle as detailed below.

i. The nitrocellulose membrane that was separated in the above step vii of section **3.7.3** was immersed in blocking buffer for one hr at 37°C.

ii. The membrane was transferred to a container having 1:100 diluted cattle convalescent serum, in PBST buffer and incubated at 37°C for one hr with gentle agitation.

iii. The membrane was washed three times using wash buffer.

iv. Enzyme tagged Protein G conjugate (1:2,000) (Dako; Denmark) prepared in PBST buffer was added and incubated at 37°C for one hr.

v. Three more similar washings of the membrane were carried out to eliminate unbound conjugate.

vi. The membrane was transferred to substrate chromogen solution and incubated in dark until the desired band intensity was achieved (about 10 min).

vii. The reaction was stopped by washing the NC membrane with excess of distilled water and then the results were documented.

Similar procedure were repeated with known negative cattle sera.

3.8 Standardisation of Indirect ELISA :

3.8.1 Buffers/ Reagents

1. Coating buffer (carbonate – bicarbonate buffer; pH 9.6)

Solution A

Sodium carbonate	:	1.06 g
D. W	:	50 ml

Solution B

Sodium bi-carbonate	:	0.84 g
D. W	:	50 ml

2. Working solution of antigen coating buffer

Sol. A.	:	7 ml
Sol. B.	:	17 ml
D. W	:	76 ml

3. Serum Dilution Buffer

Bovine gelatin	:	2 g
PBS	:	100 ml
Tween – 20	:	0.05 ml

The gelatin was dissolved in PBS by placing in a water bath at 60^{o} C for 15 to 20 min and then 0.05 ml of Tween 20 was added after taking out from the water bath.

4. Phosphate Buffer Saline (pH 7.4+0.02)

Sodium Chloride	:	7.0 g
Potassium Chloride	:	0.2g
Di-Sodium hydrogen phosphate	:	0 .353 g
Potassium di- hydrogen orthophosphate	:	1.09 g
D. W	:	1000 ml

5. Phosphate Buffer Saline Tween – 20 (PBST)

PBS (pH 7.4 +/- 0.02)	:	100 ml
Tween 20	:	0.05 ml

6. Washing buffer

PBS (pH to 7.4 +/- 0.02)	:	100 ml
D. W	:	400 ml
Tween 20	:	0.25 ml

Prepared freshly before use.

3.8.2 Stopping solution 1M H_2 So_4

Conc. H_2 So_4	:	5.5 ml
D. W.	:	94.5 ml

3.8.3 Chromogen solution

A five mg Ortho – Phenylene Diamine (OPD) tablet was dissolved in 12.5 ml of distilled water. 40 µl of three per cent H_2O_2 was added to 12.5 ml aliquot just before the use.

3.8.4 Conjugate

Rabbit anti goat IgG Horse radish peroxidase conjugate (Dako; Denmark) and enzyme tagged Protein G of 1: 8000 dilution were used.

3.8.5 ELISA Plates

Flat bottomed 96 well Nunc Maxisorp plates were used in the present study.

3.8.6 Determination of optimal dilution of *L. monocytogenes* LLO antigen and sera

3.8.6.1 Titration of *L. monocytogenes* LLO antigen

i. LLO antigen obtained in section **3.5.1** was diluted by two folds in antigen coating buffer to provide dilutions ranging from 740 ng to 0.36 ng per well. The plates were coated by transferring 100µl of each of these dilutions in to the wells in duplicate across the plate and incubated overnight at 4°C.

ii. Following day, the plates were washed three times with PBST and each time gently tapped in an inverted position against lint free absorbent.

iii. A two fold dilution of known positive and negative sera samples were made in serum dilution buffer, ranging from 1:25 to 1:200. Then, 100µl of each of these dilutions were added in duplicate to the wells and incubated at 37°C for one hour.

iv. Plates were washed as in step 2.

v. 100 µl of enzyme tagged Protein G of 1:8,000 in serum dilution buffer was added to each well and incubated at 37°C for 30 min.

vi. Plates were washed as in step ii.

vii. 100 µl of substrate chromogen was added to all the wells. Color development was observed for five to ten minutes.

viii. The enzyme substrate reaction was stopped by adding 50 µl of stopping solution.

ix. The optical density of each of the samples was recorded at 492 nm by ELISA reader (Bio-Rad).

3.8.7 Protocol of an indirect ELISA

Protocol of an indirect ELISA was standardized for the assay of antibody detection in cattle and sheep sera samples against LLO as described below.

i. An aliquot of frozen listeriolysin O was thawed and diluted to provide a suitable dilution in carbonate- bicarbonate buffer (pH 9.6) and coated on each wells by incubating at 4°C overnight**.**

ii. Following day, the plates were washed three times with PBST and each time gently tapped in an inverted position against lint free absorbent.

iii. A 100 µl of 1:50 dilutions of test sera samples in serum dilution buffer was added to each well and incubated for one hour at 37°C in an incubator.

iv. The plate was washed as in step (ii).

v. A 100 µl of 1: 8,000 dilution of protein G conjugate was added to each well and incubated for 30 min at 37°C in incubator.

vi. The plate was washed as in step (ii).

vii. 100 µl of chromogen was added to each well, color development was observed for five to ten min.

viii. 50 µl of 1 M sulphuric acid was added to each well to stop the reaction. The results were read at a wave length of 492 nm in the ELISA reader.

3.8.8 Calculation of Percent Positivity (PP) Values

The OD values of different test sera were converted to PP values for diagnostic interpretation and were calculated as follows

$$PP = \frac{\text{Replicate OD value of test serum}}{\text{Median OD value of strong positive control}} \text{ X } 100$$

3.9 Polymerase chain reaction for identification of *Listeria monocytogenes*

The PCR for the detection of *L. monocytogenes* in 80 blood samples (40 each from cattle and sheep) and PCR confirmation of reference strains was carried out using following set of primers and PCR conditions.

Equipments / reagents

a. Eppendroff Master cycler, CP2-04

b. Micropipettes (Thermo scientific, Finland)

c. PCR tubes (Axygen Inc., USA)

d. Tris - EDTA buffer (TE buffer)

3.9.1.1 Oligonucleotides Primer:

The primers for detection of *hly*A and *Lmo*2821 (Virulence specific) of *L. monocytogenes* used in this study were custom synthesized from M/G Biotech AG, Bangalore. They were reconstituted to one nmol/µl stocks in sterile TE buffer. Primers were used at a working dilution of 20 pmol/µl in sterile Nuclease Free Water (NFW). Two sets of primers one targeting *Lmo*2821 (Virulence specific) and another targeting *hly*A were used for confirmation of standard strain and for detection of *L.monocytogenes* in clinical samples respectively.The details of the primer sequences are shown in below.

(i) *Lmo*2821(Virulence specific) - 407 bp at 58^{o}C. (Shome *et al* ., 2003)

Lis 25 F: 5’ CAC CAA ATT AGA TGT GAC ACC A 3’

Lis 25 R: 5’ TAT TAT GCG TGA CAT CAA GCT C 3’

(ii) *hly*A - 210 bp at 55^{o}C (Shome *et al* ., 2003)

1F : 5’ CGC AAC AAA CTG AAG CAA AGG 3’

1R : 5’ TTG GCG GCA CAT TTG TCA 3’

3.9.1.2 Composition of PCR mix

The PCR for the detection of *L. monocytogenes* in clinical samples was carried out using LLO primers to amplify *hly*A gene yielding product of 210b . The PCR was carried out in a 25 µl reaction mixture. The PCR reaction mixture for *hly* A includes.

PCR Master mix (2X)	:	12.5 µl
F primer (20 pmole/µl)	:	0.50 µl
R primer (20 pmole/µl)	:	0.50 µl
Template DNA	:	10.00 µl
Nuclease free water	:	1.50 µl
		25.00 µl

3.9.1.3 PCR conditions for the detection of *L. monocytogenes*

The DNA amplification (Shome *et al.*, 2003) was carried out in a thermal cycler using following conditions.

Steps	Temperature	Duration	No. of cycles
Initial denaturation	93^{o}C	5 min	1
Denaturation	93^{o}C	45 sec	35
Annealing	55^{o}C/ 58^{o}C	45 sec	35
Extension	72^{o}C	1 min	35
Final extension	72^{o}C	7 min	1

The amplified products obtained were checked in 1.5 per cent agarose gel with 100 bp ladder as a marker.

3.9.1 Extraction of DNA

The DNA was extracted from 80 blood samples 40 each from cattle and sheep as per the standard protocol, using the "QIAamp DNA Mini Kit, procured from Qiagen, Germany

The kit contents include

i. Microcentrifuge tube.

ii. QIAamp spin column

iii. Proteinase K

iv. Lysis buffer (AL buffer)

v. Wash buffer ($AW_{1,}$ AW_{2})

vi. Elution buffer (AE buffer)

i. 20 µl Qiagen protease (or Proteinase K) was taken in 1.5 ml microcentrifuge tube. 200 µl of whole blood sample was added into microcentrifuge tube.

ii. 200 µl of lysis buffer (AL buffer) was added to the above, vortexed for a min and placed in 56^{o}C water bath for 15 min.

iii. The sample was given a quick spin to enable any drops attached to inner side of lid fall into microcentrifuge tube. 200 µl of ethanol (96-100 per cent) was added to sample and vortexed for 15s.

iv. Mixture was applied to QIAamp spin column and centrifuged at 8000 rpm for 1 minute, filtrate in collection tube was discarded .

v. 500 µl of wash buffer (AW_1) was added to the column and spun at 8000 rpm for one min. The contents of the collection tube were discarded and the spin column was placed back in the same collection tube.

vi. 500 µl of wash buffer (AW_2) added and spun at full speed 14,000 rpm, contents of the collection tube were discarded and the spin column was placed back in the same collection tube and centrifuged at 14,000 rpm for 1 min to eliminate chance of AW_2 carry over.

vii. Spin column placed in a new 1.5 ml microcentrifuge tube and 40 µl of elution buffer (AE buffer) added and incubated at room temperature for one min, followed by spinning at 8,000 rpm for one min for elution of DNA.

3.9.2. Spectrophotometry

The purity and concentration of genomic DNA extracted from bood samples were estimated by Spectrophotometer, Shimadzu, UV-1201 model.

3.9.3. Agarose gel electrophoresis

Equipments

a. Weighing balance (Shimazdu AX-200, Philippines Manufacturing Inc., Japan)

b. Horizontal electrophoresis apparatus with power pack (Bangalore Genei, India)

c. Microwave oven (M/s. BPL Pvt. Ltd.)

d. UV transilluminator (M/s. Pharmacia, Sweden)

e. Gel documentation unit (Alpha Imager^{TM} 2200, USA)

Reagents

a. Agarose (M/s. Bangalore Genei, Bangalore and M/s. CONDA Laboratories, Spain)

b. TBE buffer (Tris - Borate EDTA buffer) (10X, pH 8.2)

Tris base	:	108.0 g
Boric acid	:	55.0 g
EDTA disodium salt	:	8.3 g
Double distilled water up to	:	1000.0 ml

The stock solution was sterilized by autoclaving and made to 0.5X before use.

c. Gel loading dye (6X)

Bromophenol blue	:	0.25% (w/v)
Xylene cyanol	:	0.25% (w/v)
Sucrose	:	40% (w/v) in distilled water

The dye was procured from M/s. Bangalore Genei, Bangalore and stored at 4^{o}C.

d. Ethidium bromide (10 mg/ ml)

Ethidium bromide (Biogene, USA)	:	100 mg
Double distilled water	:	10 ml

The suspension was stirred to ensure that the dye was completely dissolved. The container was then wrapped in aluminum foil and stored at 4^{o}C until use.

The concentration of DNA was determined by electrophoresis on 0.8 per cent agarose as described below.

a. The edges of a clean, dry, gel casting tray was sealed at both the ends using adhesive tape. An appropriate comb was placed to form a sample slot in the gel.

b. Agarose solution was prepared by dissolving required quantity of agarose in a proportionate volume of 0.5X TBE buffer and melted in a microwave oven for one min.

c. Once the molten gel cooled, 0.5 µg of ethidium bromide per ml was added and mixed thoroughly by gentle swirling.

d. Warm agarose solution was then poured into the gel casting tray by avoiding formation of air bubbles and allowed to solidify.

e. Once agar solidified, a small amount of electrophoresis buffer was poured on the top of the gel to remove the comb. Then the buffer was poured off and the tape was removed.

f. The gel casting tray was mounted in the electrophoresis tank and the electrophoresis buffer was added just enough to cover the gel to a depth of one mm.

g. Five µl of genomic DNA was mixed with 1/6th volume of 6X gel loading dye and slowly loaded into the slots of submerged gel using a micropipette.

h. The gel tank was closed with the lid and electrical leads were connected so that the DNA would migrate towards the anode.

i. The electrophoresis was carried out at 5V/cm at room temperature until the bromophenol blue dye migrated to an appropriate distance through the gel.

j. Following electrophoresis, the gel / bands was / were visualized at 300 nm wavelength using a UV trans-illuminator and recorded in a gel documentation unit.

3.10.3 Agarose gel electrophoresis:

PCR amplified DNA was analyzed by analytical agarose gel electrophoresis as per the procedure described by Sambrook and Russell (2001). Agarose gel concentration of 1.5 per cent was prepared by boiling 0.6 gm of agarose in 40ml of 0.5 X TBE buffer to obtain a uniform molten agarose which was cast into an appropriate gel-casting tray fitted with acrylic comb and left for setting (solidification). Prior to casting the gel, the molten agarose was allowed to cool to about 50^{o}C after which ethidium bromide was added to a final concentration of 0.5µg/ml and mixed thoroughly. The acrylic comb was carefully removed after the gel had set perfectly. The tray with gel was then transferred to, and submerged in an electrophoresis tank containing 0.5 X TBE buffer. DNA to be analyzed was mixed with appropriate volume of 6X gel loading dye and charged into wells alongside 100 bp DNA ladder as a molecular weight marker. Electrophoresis was carried out at 5 V/cm until the tracking dye (Bromophenol blue) has just passed out of the gel. The DNA bands were visualized under UV illumination and documented. The molecular sizes of the DNA bands were analyzed in relation to molecular weight marker.

RESULTS

IV. RESULTS

Results obtained in the standardization and evaluation of Listeriolysin 'O' based ELISA for the serodiagnosis of listeriosis in cattle and sheep are presented here under.

4.1 Maintenance of reference strains of *Listeria monocytogenes*

Brain Heart Infusion broth was found satisfactory for the growth of reference strains of *L. monocytogenes*. Sub culturing at monthly intervals into BHI plates and slants was found optimum for the maintenance of the strains. A characteristic growth of translucent colonies with crystalline appearance in center was observed in 24-36 hr post inoculation in plates.

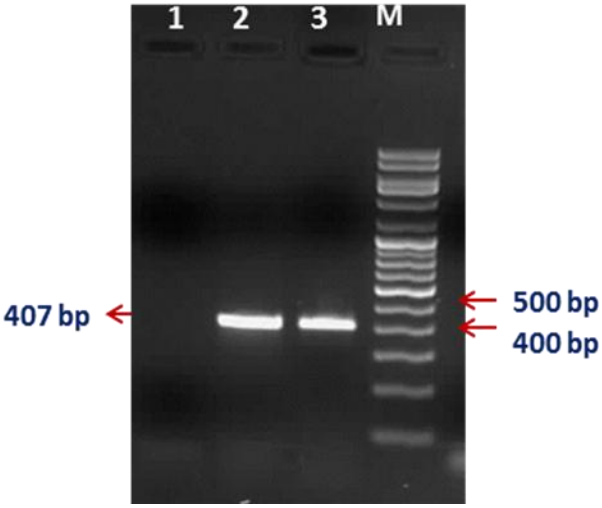
4.2 Confirmation of *L. monocytogenes* reference strain 1/2a and 4b by PCR

L. monocytogenes reference strain 1/2a and 4b grown on BHI agar was confirmed for its species using a set of primers targeting virulence specific gene *Lmo* 2821. The unique amplicons of size 407 bp were observed in lanes 2 (strain 4b) and 3 (strain1/2a) for *Lmo* 2821 gene (Fig. 1).

4.3 Isolation of *L. monocytogenes* from clinical samples

Selective enrichment of clinical samples in UVM 1 followed by UVM 2 and then streaking onto PALCAM agar plates was found to support growth of Listeria species. However, in the present study, out of 140 samples from cattle (90) and sheep (50); only one each of 40 blood samples from cattle and sheep and none of the mastitis milk samples and vaginal swabs yielded growth on PALCAM plates suggestive of Listeria spp. (Plate 1) Turbidity in UVM broth was observed within 24 hrs post inoculation.

Fig 1. Agarose gel electrophoresis of PCR amplified products of *Lmo* 2821 gene of *L. monocytogenes* reference strains

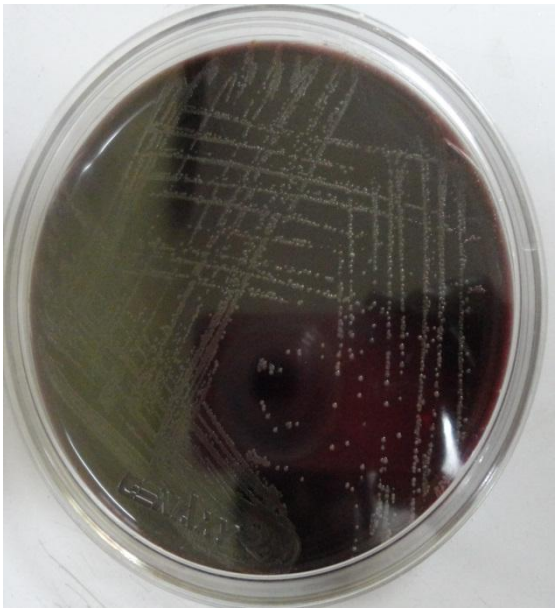


Lane 2 - *L. monocytogenes* reference strain strain 4b

Lane 3 - *L. monocytogenes* rernce strain strain 1/2a

Lane M – 100 bp DNA Ladder

Plate 1. Characteristic colonies of *Listeria* spp. on PALCAM agar plate



Characteristic grey green color colonies surrounded by a black zone of aesculin hydrolysis against cherry red background on PALCAM agar by 36-48 hr post inoculation were considered as *Listeria* species. A number of these presumptive colonies from blood samples were stored in BHI slants. In all, two isolates were confirmed as *L. monocytogenes*, by biochemical characterization using HiListeria identification kit (table 2 and 3) with a cultural positivity of 1.1 and 2 per cent respectively in cattle and sheep. Both the isolates were from blood samples belonged to Maharashtra state one each of cattle and sheep with history of abortion and reproductive disorders. The isolates were either preserved in glycerol stock of BHI broth or maintained in the laboratory by periodic sub-culturing.

4.4 Extraction and purification of Listeriolysin 'O' antigen

Extraction of Listeriolysin 'O' antigen from 500 ml of cell free supernatant of an 18 hr old growth of *L. monocytogenes* (1/2a) in BHI broth at 37°C, yielded 10 ml of crude extract, which .was equilibrated to pH 6 by overnight dialysis in Tris-HCl pH 6 as described in materials and method section of 3.5.

Purification of this crude extract by ion exchange chromatography using DEAE cellulose column equilibrated to pH 6 with Tris HCl, has produced five major peaks giving absorbance at 280 nm and was pooled accordingly into five different fractions. Fractions were then concentrated using sucrose into one ml each and 1mM of PMSF was added and stored at – 20^{o} C.

Table 2. Isolation of *L. monocytogenes* from clinical samples

Species	No. of samples tested	*L. monocytogenes* isolated
Cattle	40 (Blood)	1
	40 (Mastitis milk)	-
	10 (Vaginal swabs)	-
	Total 90	1
Sheep	40 (Blood)	1
	10 (Vaginal swabs)	-
	Total 50	1

Table 3. Details of biochemical tests employed for the identification of *L. monocytogenes*

Test	Positive Reaction	Negative Reaction
Catalase	Effervescence with 3 per cent H_2O_2	-
Nitrate Reduction	-	Colourless
Esculin Hydrolysis	Black	-
Voges Prokauer's	Pinkish red	-
Methyl Red	Red	-
Xylose fermentation	-	Pink
Lactose fermentation	-	Pink
Glucose fermentation	Yellow	-
α-Methyl Mannoside	Yellow	-
Rhamnose	Yellow	-
Ribose	-	Orangish Red
Mannitol	-	Pink

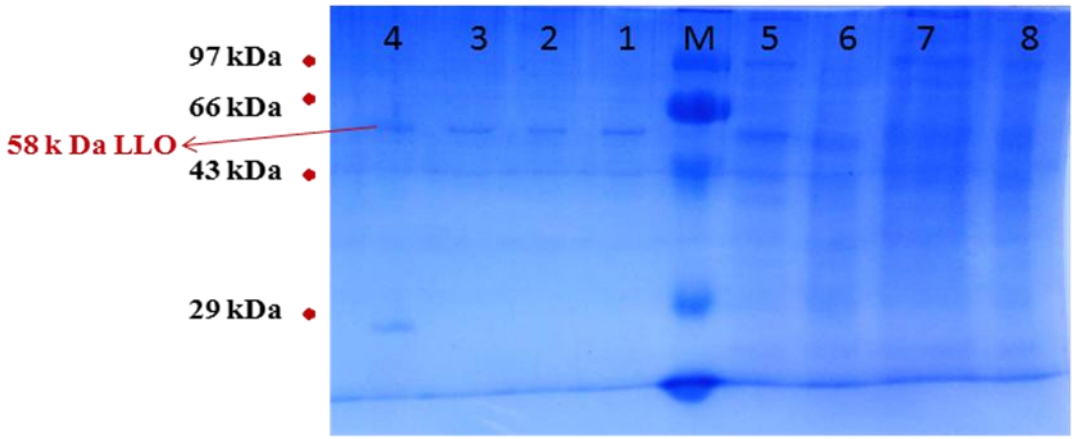
4.4.1 SDS-PAGE analysis of crude and column purified proteins.

The crude extract of protein and five different fractions of proteins obtained after DEAE purification were analyzed by 10 per cent SDS PAGE along with the medium sized protein molecular weight marker. The Coomassie Brilliant Blue R250 (CBB) stained gel showed a distinct band of LLO with a molecular mass at about 58 kDa, in between 43 and 66 kDa protein molecular weight markers. This distinct band was seen in the first three purified fractions of the protein samples (Fig. 2; lanes 1 to 3), whereas, fourth fraction yielded an additional band at about 20 kda (lane 4) and fifth fraction did not yield any distinct bands. However, besides a 58 kDa protein, several other protein bands could be seen in the crude extracts (Fig. 2; lanes 6 to 8).

4.4.2 Confirmation of protein by Western blot analysis.

A distinct band of 58 kDa protein resolved in SDS-PAGE was further analysed by Western blotting for its immunogenic property against both convalescent and known negative sera from cattle. Upon transfer of the proteins onto Nitrocellulose (NC) membrane and probing with the convalescent serum, a distinct band was seen corresponding to 58 kDa protein, the listeriolysin O (Fig. 3; lanes 1 to 4). However, on probing with negative serum, no band could be seen (Fig. 3).

Fig 2. SDS- PAGE characterisation of purified Listeriolysin 'O' and crude extract.



Lanes 1 to 3 - First three fractions of DEAE cellulose column chromatography with purified Listeriolysin 'O' protein.

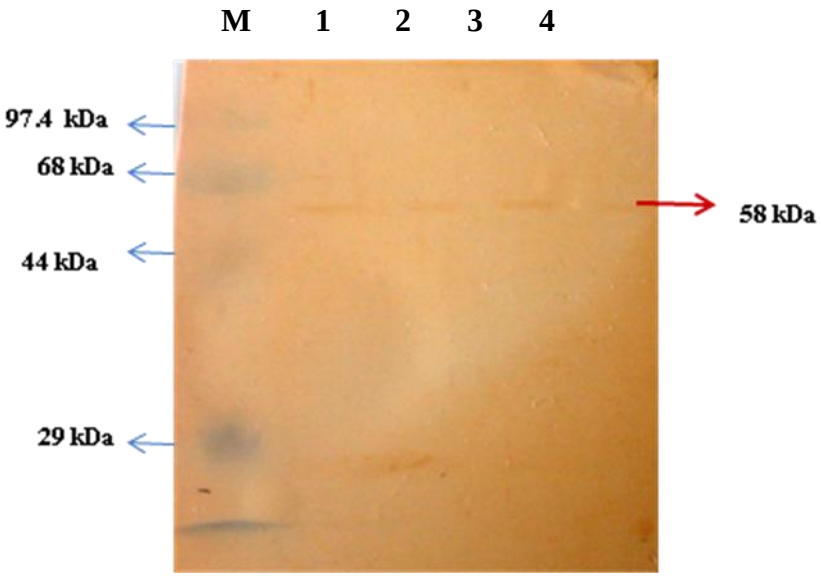
Lane M - Protein molecular weight marker

Lane 4 - Fourth fraction obtained during DEAE cellulose column chromatography.

Lane 5 - Fifth fraction obtained during DEAE cellulose column chromatography.

Lanes 6 to 8 - Crude protein extracts

Fig 3. Confirmation of Listeriolysin 'O' by western blot analysis.



Lane M – Protein molecular weight marker (Medium range).

Lanes 1 to 4 - Purified Listeriolysin 'O' protein probed with known convalescent serum of cattle.

4.5 Standardization of Indirect ELISA

4.5.1 Determination of optimal dilution of *L. monocytogenes* LLO antigen and sera

The optical density values obtained with varying concentrations of *L. monocytogenes* LLO antigen against different dilutions of known positive cattle sera are presented in Table 4.There is a sharp fall in OD values beyond a protein concentration of 190 ng per well with a dilution of 1:50 of known positive cattle sera. Therefore, 190 ng protein per well with a serum dilution of 1:50 was used as working dilutions in the present study (Table 4).

4.5.2 Determination of optimal dilution of conjugate

The protein G HRPO conjugate serially diluted from 1:1000 to 1:10,000 was titrated against a 1:50 dilution of known positive cattle serum. A 1:1000 dilution of conjugate gave an OD of 1.4 while the end dilution 1:10,000 showed an OD of 0.67 (Fig. 4). Therefore, a 1:8000 dilution of conjugate giving an optimum OD 1.0 was used as a working dilution in the present study.

4.5.3 Determination of cut – off point

The frequency distribution of Per cent Positivity (PP) values of 235 cattle and 252 sheep sera samples obtained in indirect ELISA is illustrated in Fig. 5 and 6. There is a sharp decline noticed in the frequency distribution curve at a PP of 70 for cattle. Accordingly, samples showing PP values of more than 70 were considered as positive, between 60 to 70 were taken as doubtful and less than 60 as negative. Whereas, the decline was at 65 for sheep and accordingly, samples showing PP values of more than 65 were considered as positive, between 55 to 65 as doubtful and less than 65 as negatives.

Table 4. **Determination of optimal dilution of *L. monocytogenes* LLO antigen and sera**

Dilution of antigen (ng/100µl/well)	OD values of different dilutions of known positive cattle serum at 492 nm			
	1:25	**1:50**	**1:100**	**1:200**
1:50 (740)	1.32	1.23	0.53	0.18
1:100 (370)	1.25	1.10	0.46	0.16
1:200 (190)	1.17	**1.01**	0.41	0.15
1:400 (90)	0.96	0.86	0.34	0.13
1:800 (50)	0.73	0.61	0.23	0.109
1:1600 (25)	0.48	0.37	0.20	0.102
1:3200 (12.5)	0.44	0.29	0.18	0.10
1:6400 (6.25)	0.40	0.20	0.10	0.10

Fig. 4. Determination of optimal dilution of protein G HRPO conjugate.

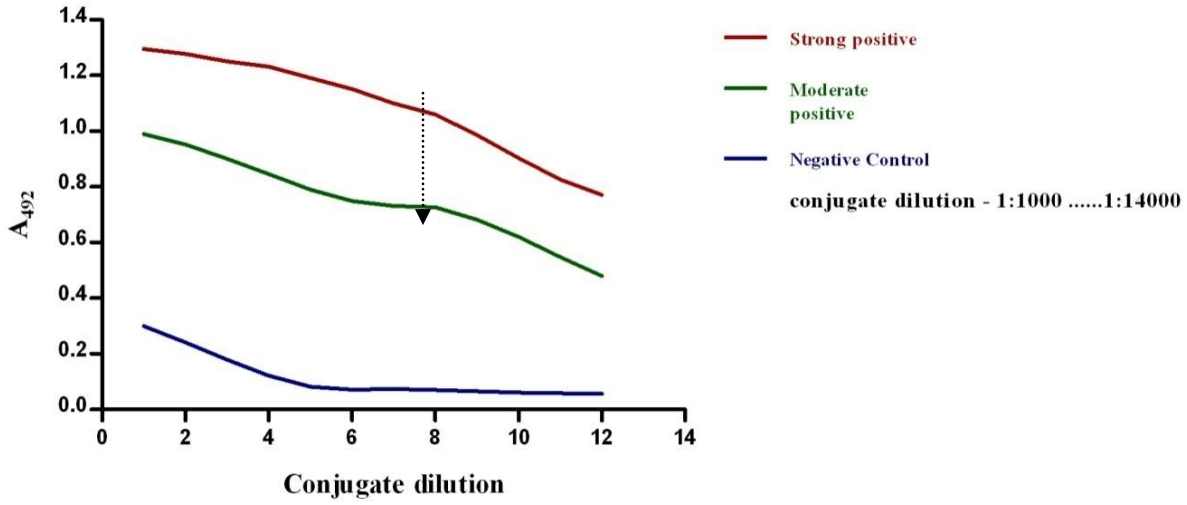


Fig 5. Frequency distribution of PP values of 235 cattle sera samples against Listeriolysin 'O' as detected by indirect ELISA

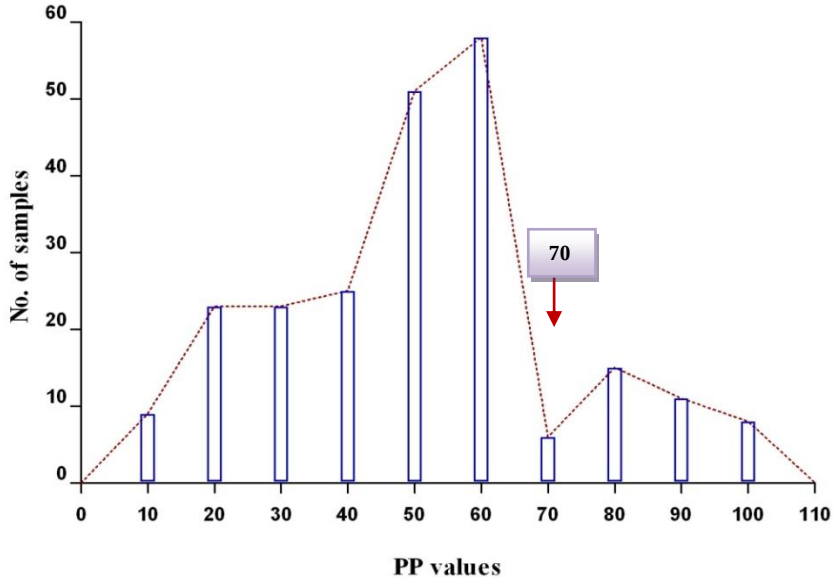
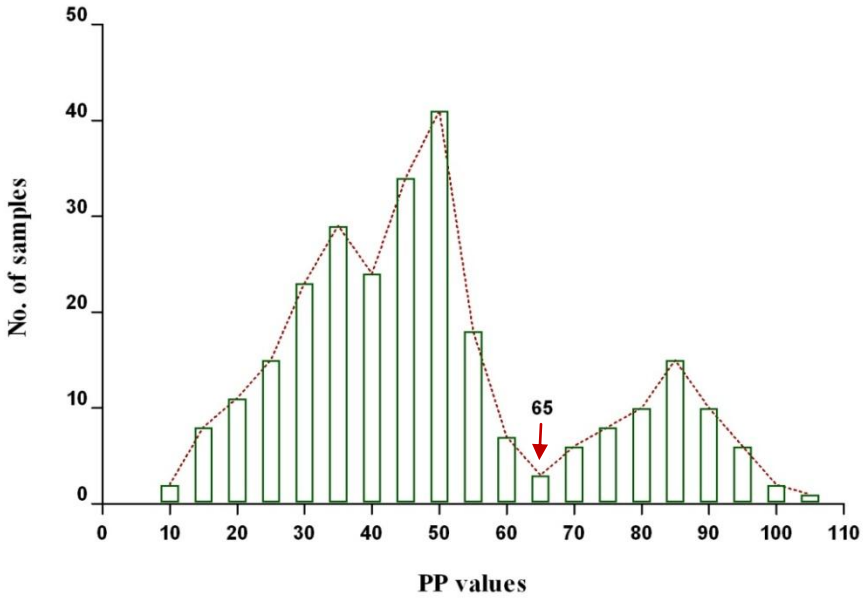


Fig 6. Frequency distribution of PP values of 252 Sheep sera samples against Listeriolysin 'O' as detected by indirect ELISA



4.5.4 Sreening of serum samples by ELISA

In all, 18.3 per cent (44 out of 235) of cattle and 23 per cent (59 out of 252) of sheep serum samples tested were declared positives in indirect ELISA. Meanwhile 26.3 per cent of cattle and 23.8 per cent of sheep serum samples are doubtful and remaining 54.8 per cent of cattle and 52.7 per cent sheep serum samples were considered negative and region wise results are set out in Table 5.

4.6 Polymerase chain reaction for identification of *L. monocytogenes*

4.6.1 Extraction of Genomic DNA

Listerial DNA from blood samples was extracted using commercially available kits procured from Qiagen, Germany. The purity and concentration of DNA samples were analyzed by UV spectrophotometer at an absorbance 260 and 280 nm and agarose gel electrophoresis (0.8% w/v). Upon electrophoresis and ethidium bromide staining, the genomic DNA samples were visualized as high molecular size distinct bands under UV illumination. In the UV spectrophotometer analysis, A_{260}/A_{280} ratio was found to be between 1.76 and 1.94 for the DNA samples.

4.6.2 Detection of *L. monocytogenes* from clinical samples by PCR

Listeria monocytogenes in clinical samples was detected by PCR assay targeting *hly*A gene yielding 210 bp size product at annealing temperature of 58^{o}C. The genomic DNA extracted from 80 blood samples from cattle and sheep were used as template for amplifying above genes of *L. monocytogenes* with a set of published primer sequences as mentioned in materials and methods section 3.9. The PCR conditions described in section 3.10.2 were employed.

Table 5. State wise distribution of cattle and sheep sera samples positive for Listeriosis by indirect ELISA

State	Cattle		Sheep	
	No. of Sera Samples Tested	No. of positives	No. of Sera Samples Tested	No. of Positives
Karnataka	65	13	80	22
Andhra Pradesh	-	-	17	4
Tamil nadu	8	2	-	-
Maharashtra	80	24	33	9
Orissa	-	-	33	2
Rajasthan	40	3	14	2
Gujarat	-	-	43	16
Manipur	32	2	32	4
Total	235	44 (18.3%)	252	59 (23%)

The products were analyzed by 1.5 per cent agarose gel electrophoresis alongside 100 bp DNA ladder. The unique amplicons of size 210 bp were observed in lanes 1, 2 and 3 (Fig.7) indicating the presence of bacterial target sequence corresponding to *hly*A gene. Out of 40 cattle blood samples one blood sample from Maharashtra (lane 3) and two (lane 1, 2) of the 40 sheep blood samples were positive for *L. monocytogenes* yielding positivity rate of 2.5 per cent and 5 per cent respectively (Table 6).

4.7 Comparision of isolation of *L. monocytogenes* and indirect ELISA

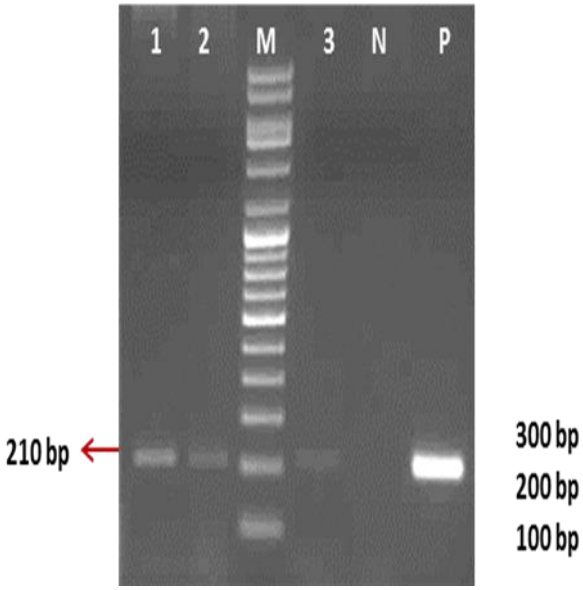
Cultural isolation of *L. monocytogenes* and detection of anti-listeriolysin O (ALLO) antibodies by indirect ELISA was compared for 40 common blood samples for cattle and sheep separately. Listeriolysin O-based indirect-ELISA did not agree with the isolation *of L. monocytogenes* from blood. Anti-listeriolysin O antibodies were detected in both cattle and sheep which were culturally positive for *L. monocytogenes*. However, out of 39 culture negative cattle, 8 (20.5%) were found seropositive for ALLO, whereas, 31 (79.4%) were negative (Table 7). Similarly, out of 39 culture negative sheep, 11 (28.2%) were found to be seropositive for ALLO while, remaining 28 (71.7%) were negative (Table 8).

4.8 Evaluation of ALLO ELISA with PCR and Isolation

Listeriolysin O based indirect ELISA of cattle (40) and sheep (40) were compared with PCR and isolation (Table 9). For which, 40 each of the blood samples from cattle and sheep were processed for isolation and PCR assay targeting *hly*A gene and respective sera from same animals were included for LLO ELISA. Only one of nine cattle seropositive for ALLO was positive by both isolation and PCR, whereas remaining

eight (20.5 %) seropositive for ALLO were culture and PCR negative. The rest of the 31 (79.4%) of 40 cattle samples seronegative for ALLO were culture and PCR negative as well. Similarly of 12 sheep samples seropositive for ALLO, only one was positive by bacterial isolation where as two were PCR positive. While, remaining 28 (71.7 %) of 40 samples compared, were ALLO as well as isolation and PCR negative.

Fig 7. Agarose gel electrophoresis of PCR amplified products of gene *hly*A *of L. monocytogenes* from clinical samples.



Lane 1 and 2 – sheep blood samples from Maharasthra.

LaneM - 100 bp DNA Ladder.

Lane 3 – cattle blood sample from Maharasthra.

Lane N – Negative control.

Lane P – Positive control

Table 6. Screening of cattle and sheep blood samples for *L.monocytogenes* by *hly*A specific PCR

Species	Region	No. tested	No. positive
Cattle	Maharastra	20 (blood)	1
	Karnataka	20 (blood)	-
	Total	40	1(2.5 %)
Sheep	Maharastra	20 (blood)	2
	Karnataka	20 (blood)	-
	Total	40	2 (5%)

Table 7. Seropositivity for anti-listeriolysin O antibodies in cattle by indirect ELISA (N=40)

Culture status	No. of samples		Total
	ALLO Positive	ALLO Negative	
Positive	1	-	1
Negative	8 (20.5%)	31(79.4%)	39
Total	9 (22.5%)	31(77.5%)	40

Table 8 Seropositivity for anti-listeriolysin O antibodies in sheep by indirect ELISA (N=40)

Culture status	No. of samples		Total
	ALLO Positive	ALLO Negative	
Positive	1	-	1
Negative	11 (28.2%)	28 (71.7%)	39
Total	12 (30%)	28 (70%)	40

Table 9. Evaluation of ALLO ELISA with PCR and isolation

Species	No. tested	Culture	ELISA	PCR
Cattle				
Maharastra	20	1	7	1
Karnataka	20	-	2	-
Total	40	1(2.5%)	9 (22.5 %)	1(2.5%)
Sheep				
Maharastra	20	1	9	2
Karnataka	20	-	3	-
Total	40	1(2.5 %)	12 (30 %)	2 (5 %)

DISCUSSION

V. DISCUSSION

Listeria monocytogenes is an important intracellular bacterial pathogen causing listeriosis in variety of domestic animals including man without any particular host preponderance. Cases of listeriosis arise mainly from the ingestion of contaminated food and the disease is particularly common in ruminants fed on silage. (Low and Donachie, 1997). Clinical listeriosis occurs usually, secondary to management changes such as silage feeding, winter housing, and increased environmental contamination due to more confined housing.

Listeria monocytogenes is responsible for, about 98 per cent of human listeriosis and 85 per cent of animal cases (Rajeev Kumar, 2011) though, rarely *L. seeligeri* and *L. ivanovii* have been implicated with abortions in sheep. Although the morbidity of listeriosis is relatively low, the mortality of the systemic/encephalitic disease can be very high, with values in the vicinity of 30 per cent (Mathakiya *et al.,* 2011), which imposes the application of the new diagnostic and screening techniques for *L. monocytogenes* such as serological detection of antilisterial antibodies.

5.1 Scenario of Listeriosis

Listeriosis is one of the important bacterial diseases of animals with a broad distribution; 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).

Reports of listeriosis from animals and humans in India are scanty, either because of failure to identify the isolate, its rarity, improper isolation techniques, low incidence rate or lack of awareness. The epidemiological data available in the country to date is neither adequate nor reliable for assessing the extent of infection in human beings and animals; since, whatever the data available is based on mere conventional diagnostic tests. Though, these conventional bacteriological tests and histological examination are time consuming and lacks specificity, but they are still considered as the ‘‘gold standard’ to which other methods are compared.

Traditional identification methods involving culture methods based on selective enrichment and plating followed by the characterization of Listeria spp. based on colony morphology, sugar fermentation and haemolytic properties are lengthy, as a result more rapid tests are developed based on serology or employing molecular techniques with varied methods of protein extraction and concentration for the purpose of antigen to be used in ELISA and DNA extraction methods for PCR respectively. Immunochemical technology, such as immuno-magnetic beads to extract the targeted bacterial cells and also use of DNA probes to concentrate the DNA material and extract it from the samples have been frequently used. Another technique that is commonly used is the chemical extraction and purification of the DNA before PCR analysis. By incorporating these methods and techniques together, rapid detection of *L. monocytogenes* can become a very specific and highly sensitive level (Hudson *et al*., 2001).

5.2 Media for the maintenance of reference strains of *Listeria monocytogenes*

Brain Heart Infusion media used in the present study was found satisfactory for the maintenance of reference strains of *L. monocytogenes*. A characteristic growth of translucent colonies with crystalline appearance in center was observed in 24-36 hr post inoculation in plates.

5.3 PCR for the Confirmation of *L. monocytogenes* reference strain 1/2a and 4b

In the absence of a reliable molecular method for the detection of virulent *L. monocytogenes*, PCR detection of known *L. monocytogenes* virulence genes, such as *inl*A, *inl*B, *act*A, *hly*A, *plc*A and *plc*B, has not proven suitable for differentiation of virulent and avirulent isolates as these genes are consistently found in *L. monocytogenes*. One of the putative internalin genes, *lmo*2821was detected in most *L. monocytogenes* strains that were considered to be virulent, suggesting that virulent *L. monocytogenes* strains possess genes that are not present in avirulent isolates, and it could serve as markers for PCR assessment of virulent *L. monocytogenes* (Liu *et al*., 2003).

In present study, confirmation of *L. monocytogenes* reference strain 1/2a and 4b for its species by *in vitro* DNA amplification using set of primer targeting virulence specific gene *lmo* 2821 was found to be very specific. The primers and PCR conditions employed yielded specific amplicons of 407 bp.

Use of internalin gene *lmo*2821 as a virulent marker and to classify the *L. monocytogenes* serotype 4b strains into different lineages was also reported by Liu *et al*. (2006). They made use of species specific *lmo*0733 and virulence specific

*lmo*2821primers. The virulence specific *lmo*2821primer detected all *L. monocytogenes* except the strains of 4a serotype and two strains of serotype 4b lineage III. Southern blot analysis using species-specific *lmo* 0733 and virulence-specific *lmo* 2821 gene probes largely confirmed the PCR results. PCR assays for various *L. monocytogenes* serotypes are proved to be rapid, sensitive and specific tests for the identification of *L. monocytogenes* which allows appropriate antibiotic therapy. Furthermore, development of typing procedures to trace *L. monocytogenes* strains involved in disease outbreaks will help limit the spread of the disease.

5.4 Isolation of *L. monocytogenes* from clinical samples

Selective enrichment of clinical samples in UVM 1 followed by UVM 2 and then streaking onto PALCAM agar plates was found to be satisfactory, as it yielded characteristic grey green color colonies surrounded by a black zone of aesculin hydrolysis against cherry red background on PALCAM agar by 36-48 hrs post inoculation. Conventional bacteriological methods are disadvantageous owing to their relatively long period of processing, several 'hands on' manipulations, for the requirement for many different chemicals, reagents and media and also the possibility of contaminating microorganisms masking the presence of the target ones. The use of alternative enrichment procedures and culture media incorporated with various selective agents such as lithium chloride, polymixin B, acriflavine, nalidixic acid and ceftazidime that allow growth of *L. monocytogenes* at normal incubation temperatures have shortened the time required for selective growth of the organism.

In the present study, out of 140 samples from cattle (90) and sheep (50) processed for isolation, only one each of 40 blood samples from cattle and sheep and none of the mastitis milk samples, vaginal swabs yielded growth on PALCAM plates suggestive of Listeria *spp* and isolates were confirmed by panel of tests as per HiListeria identification kit with cultural positivity of 1.1 and 2 per cent respectively in cattle and sheep. This finding is in agreement with Takashi *et al*. (1991) who reported a prevalence of *L. monocytogenes* to be 1.9 per cent in fecal samples of cattle in Japan by using combination of oxoid and LPM media after cold enrichment in PBS at 4^{o}C for 4 to 6 weeks.

The isolation rate of pathogenic *L. monocytogenes* from blood samples is in accordance with our earlier workers wherein 1.53 per cent of the blood samples collected from slaughtered buffaloes were found positive for pathogenic *L. Monocytogenes* (Chaudhari *et al.,* 2001) and *L. monocytogenes* could be isolated from two (1.6%) buffaloe blood samples collected from Bareilly (Chaudhari *et al*., 2004). The low recovery rate of the pathogen from blood observed in this study is in accordance with the observations made by other workers (Low and Donachie 1997 and Chaudhari *et al.,* 2004) that septicemia in listeriosis was relatively uncommon, although occasional massive outbreaks were linked to it.

Pathogenic *L. monocytogenes* could not be isolated from the vaginal samples collected from cattle and sheep. The published work on the recovery of *L.monocytogenes* from vaginal samples of cattle is largely lacking. However Sharma *et al.* (1996) isolated *L. monocytogenes* from vaginal samples of sheep (3.7 %) and goats (3.32 %). Whereas, Simranpreet *et al*. (2010) recovered only one pathogenic *L. ivanovii* from a genital swab

of a sheep out of 333 faecal, genital swab and blood samples from 111 animals in total (cattle, buffaloes, sheep and goats) and pathogenic *L. ivanovii* was also isolated from 4.68 to 8.69 per cent of vaginal samples collected from cases of repeat breeding and abortion in cows, respectively (Thakur, 2000).

The *L. monocytogenes* could not be isolated from the bovine raw milk samples, however Bhilegaonkar *et al.* (1997) reported the prevalence of 8.1 per cent of the 86 bovine raw milk samples tested in Northern India and Barbuddhe *et al*. (2002) reported 6.25 per cent and 26.13 per cent prevalence of *L. monocytogenes* and Listeria *spp.*, respectively in 64 raw buffalo milk sampled in Northern India. The isolates were of non-pathogenic species, with *L. grayi* predominating. The incidence of contaminated milk samples also varies among countries, being 1.2 per cent of 1,132,958 raw milk samples in Denmark (Jensen *et al*., 1996), 3.62 per cent of 774 samples in Spain (Gaya *et al*., 1998). Of 1300 raw milk samples from bulk tanks at dairy farms in Mexico City, 13 per cent of were positive for *L. monocytogenes*; six per cent for *L. ivanovii*; four per cent for *L. seeligeri* and one per cent for *L. innocua* (Carlos *et al*., 2001). The variation in the number of Listeria spp. from different studies carried out could also be due to the diverse isolation and enumeration methods. The sources of Listeria spp. in raw milk have been reported to be faecal and environmental contamination during milking, storage and transport, infected cows in dairy farms and poor silage quality (Kalorey *et al.,* 2008) and even Menzies *et al*. (2000) described *L. monocytogenes* as a “less common cause” of bovine environmental mastitis and may often be associated with the accidental introduction of the organism during intra-mammary infusion.

The lower occurrence of listeric mastitis in cattle is also supported by findings of Fedio *et al*. (1990) who found that only one milking cow out of 72 animals infected with subclinical mastitis was caused by *L. monocytogenes* serotype 1. They opined that *L. monocytogenes* is not an invasive organism for the bovine udder even with a rather serious mastitis problem. However poor milking practices can predisposes the udder infection in presence of *L. monocytogenes* in the environment (feces and feed). They concluded that mastitis due to *L. monocytogenes* is rare and occurs probably under conditions conducive to all forms of udder infections. Mohammed *et al*. (2009b) was also of the same opinion, wherein they elucidated the ecology of *L. monocytogenes* on dairy cattle farms in central New York State, by determining the prevalence of the organism in various samples and concluded that *L monocytogenes* was more prevalent in samples obtained from dairy cattle and their environment than in milk samples. Further *L. monocytogenes* may directly contaminate milk as a consequence of listerial mastitis, encephalitis or Listeria related abortion in cattle and asymptomatic cows can also shed *L. monocytogenes* in their milk for many months.

The overall occurrence of *L. monocytogenes* (0.55 per cent of 243 cattle and buffaloes) with subclinical mastitis was reported by Rawool *et al*. (2007). Considering the level of incidence of *L. monocytogenes* in dairy cows, it seems likely that *L. monocytogenes* may be transferred to humans or to milk products by raw milk or milk that have not been correctly pasteurized or that have been contaminated post pasteurization with raw milk and it is also 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. 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 (Buffalo Bulletin, 2010). Hence, it is necessary to maintain strict hygiene and practice of pasteurization of raw milk besides regular screening of dairy cows for mastitis in order to minimize the human health risk.

5.5 Extraction and purification of Listeriolysin 'O' antigen

Listeriolysin 'O' is a 58-kDa protein belonging to the group of SH-activated exotoxin, serves as a crucial virulence factor for bacterial evasion produced by all pathogenic strains of *L. monocytogenes*. It is also the target for specific antibodies by inducing T-cell recognition during the course of an acute listeric infection in human patients (Berche *et al*., 1990). Various researchers have also reported that antibodies to LLO are reliable indicators for serodiagnosis of listeriosis in farm animals experimentally infected with *L.* monocytogenes, such as lambs (Low and Donachie, 1991), goats (Miettinen *et al*., 1990) and cattle (Baetz and Wesley, 1995) respectively. These observations were taken into consideration to design an useful sero-diagnostic method for listeriosis in present study using purified listeriolysin O as the test antigen.

The crude extract of Listeriolysin 'O' was purified by DEAE ion exchange chromatography. Diethyl aminoethyl cellulose is a positively charged resin used in ion exchange column chromatography, to purify proteins based on the charge of the protein. Gel matrix beads are derivatized with diethylaminoethanol (DEAE), tertiary amine functional group which binds to negatively charged proteins on the matrix, until released by increasing the salt concentration of the solvent.

The extraction of Listeriolysin 'O' antigen from cell free culture supernatant of *L. monocytogenes* (1/2a) grown in BHI and the purification of crude extract by ion exchange chromatography using DEAE cellulose column is in accordance with Lhopital *et al*. (1993) and Shakuntala *et al*. (2006). The purification method for listeriolysin O was efficient and SDS PAGE characterization of purified protein fractions was shown in as single homogeneous band with an approximate molecular weight of 58-kDa in first three fractions which remained immuno reactive as confirmed by western blotting using listeria convalescent cattle sera and bound efficiently to polystyrene microtitre plate.

The immuno reactivity of purified listeriolysin O by immunoblotting against convalescent antiserum of sheep and hyperimmune anti-LLO rabbit serum was tested by Low *et al.* (1992) further they developed an indirect enzyme-linked immunosorbent assay for the diagnosis of Listeric infections in sheep. The kinetics of antibody production against purified listeriolysin O was analysed by dot blot assay by Lhopital *et al*. (1993), following an oral infection of sheep with *L. monocytogenes*. They reported that antibodies to LLO are constantly produced during oral infection even with a low infective dose with specific antibodies appearing as soon as day nine of an oral infection and peaked by day 20 of infection, confirming that LLO is highly immunogenic and induces a strong humoral immune response during infection.

Besides, Gholizadeh *et al*. (1996) evaluated the use of recombinant LLO expressed in *E. coli* as test antigen in a Western blot assay and found that all twenty one serum samples obtained from human patients with listeriosis were found positive for antibodies to listeriolysin O (ALLO) by a reference dot blot test and only one of 100

serum samples from healthy adults reacted with recombinant LLO and opined that recombinant LLO as a specific and sensitive antigen for the detection of ALLO.

5.6 Standardization of Indirect ELISA

An antigen concentration of 190 ng per well giving an approximate optical density of about one at 492 nm and 1:50 dilution of known positive cattle sera was found to be optimum and were employed as working strengths in the present study. Boerlin *et al*. (2003) also used 200 ng per well of recombinant listeriolysin O and internalin A antigens with 1 in 200 dilution of serum, for the specific detection of anti *L. monocytogenes* antibodies in cattle. However, there are reports of using, much lower concentration of antigen, 40 ng per well by Shakuntala *et al*. (2006) and 25 ng per by Low *et al*. (1992) who optimized the concentration of purified LLO for use in the indirect ELISA against a series of convalescent antiserum dilutions. They reported A_{492} values in the range of 0.2 to 1.4 with serum dilutions of 1:200 to 1:800 for positive control sera. The log dose-response curve was linear for each antibody tested and concluded that for each serum, increasing antibody dilution was shown to had less effect on absorbance than decreasing the antigen coated based on which experimental sera were tested by indirect ELISA with antigen coating of 25 ng per well and using a 1:400 dilution of antibody and even Baetz *et al*. (1996) used 25 ng LLO per well and 1:100 dilution of test serum to detect listeriosis in dairy cattle experimentally infected with *L. monocytogenes*.

Similarly, a 1:8,000 dilution of protein G HRPO conjugate was found optimum in this study. There are several other reports of using anti-bovine HRPO conjugate in

various dilutions; 1:2000 by Shakuntala *et al.* (2006); 1:5000 by Baetz and Wesley (1995) and 1:5000 by Rathod *et al.* (2008). The higher dilution of conjugate utilized in the present study to obtain an optimum OD was probably an indication of better quality of antigen employed in the test besides being an economical one.

The carbonate – bicarbonate coating buffer (pH 9.6) used in this study was found to provide optimum pH and ionic strength for antigen coating and similar observations were also made by Boerlin *et al.* (2003) and Rathod *et al.* (2008).

The serum dilution buffer consisting of two per cent bovine gelatin with PBS Tween – 20 (0.05%) was employed to block any non-specific binding, was found to be satisfactory as there was minimum background activity in conjugate control and negative control wells. There are also reports of using PBS for the same purpose by Rathod *et al.* (2008) and Shakuntala *et al.* (2006).

The washing buffer comprised of PBS supplemented with 0.25 per cent Tween - 20 was found to be optimal in inhibiting non-specific hydrophobic binding to the plate surface, while PBS with 0.05 per cent was employed by Rathod *et al.* (2008) and Shakuntala *et al.* (2006). However NaCl-Tween solution (0.05%, v/v Tween 20 in 9 g l^{-1} NaCl) was reported to be used by Zundel *et al.* (2007) as washing buffer with satisfactory results.

5.6.1 Determination of cut-off point

The PP values of 235 cattle and 252 sheep sera samples when plotted separately in a graph, showed a distinct separation at PP value of 70 and 65 for cattle and sheep

respectively. At these points, there was a clear differentiation of cattle and sheep having significant quantity of antibodies on account of infection from the animals having lower antibody titers were considered as negative in this indirect ELISA.

The conversion of OD values to PP values offers advantages, as it obviates the day to day variations arising out of OD values of test samples in comparison with control values of each plate. The available literature on this aspect revealed that some of the researchers concluded their results based on serum sample showing a positive to negative (P/N) ratio of more than two at a serum dilution of 1:200 as positive (Barbuddhe *et al*. 2000a, Chaudhari *et al*. 2004, Bhanu Rekha *et al.* 2006 and Shakuntala *et al*. 2006). The results interpreted in the present study based on PP values offer a means to distinctly separate animals which are positive or negative.

In the present study, a higher positivity for anti- LLO antibodies (ALLO) was detected by indirect-ELISA in apparently healthy cattle (18.3 %) and sheep (23 %) could be explained in the light of published reports indicating sero-conversion to ALLO after oral or subcutaneous infection in sheep (Low and Donachie, 1991 and Lhopital *et al.,* 1993). The high positivity for ALLO in the absence of any clinical disease observed in our study may possibly be attributed to exposure of these animals to low infective doses which has been reported to elicit persistent immune response to LLO equal to that with higher infective doses (Lhopital *et al.,* 1993) through various established sources of infection including poor quality silage and vegetables (Low *et al.,* 1992). However the seropositivity for ALLO is less than that of earlier reports as 40 per cent in buffaloes with various reproductive disorders (Shakuntala *et al*., 2006) and 41.13 per cent by LLO-

based indirect ELISA (Barbuddhe *et al*., 2000a) in apparently healthy goats. These variations might be attributed to several environmental and host factors.

In the present study, evaluation of indirect ELISA with isolation and *hly*A PCR for 40 common cattle and sheep samples separately showed that, ELISA was not in agreement with the cultural isolation or PCR. Anti- LLO antibodies was also detected in eight (20.5%) and eleven (28.2%) of the serum samples from culturally negative cattle and sheep respectively. Kinetics of ALLO production following experimental infection with pathogenic *L. monocytogenes* of sheep (Lhopital *et al.,* 1993), goats (Miettinen *et al.,* 1990 and Bhanu Rekha, 2006) and buffaloes (Chaudhari *et al.*, 2001) showed that faecal, nasal and blood cultures became negative for the pathogen as ALLO peaked in the sera of infected animals.

In the present study, out of nine cattle seropositive for ALLO, the *hlyA* gene was detected in only one of the cattle sample and similarly two sheep samples out of 12 were PCR positive whereas all other animal samples were negative in PCR analysis. This could be explained by observations of Amagliani *et al*. (2006) that in the early phase of the infection frequent PCR positive reactions were due to the shedding of bacteria in the samples but the corresponding serum samples were generally ELISA negative because of the low level of circulating antibodies. On the contrary, in later stages and after antibiotic treatment of the animals, a lower PCR positivity rate and a high seroprevalence rate were observed. Such findings also underline the fact that the serological test alone is not sufficient for the diagnosis of listeriosis. However, it can still be considered a useful tool

for a quick and not expensive processing of large number of samples (Amagliani *et al*., 2006).

The usefulness of a combination of PCR and ELISA tests as a diagnostic mass-screening tool was evaluated by Amagliani *et al*. (2006). The assays were carried out with two commercial kits detecting a species-specific DNA sequence the *hly*A gene, and anti-LLO IgG, respectively in symptomatic and asymptomatic sheep flocks. The Polymerase chain reaction amplified 172 bp gene fragment from milk DNA samples yielding positivity at the rate of 47 per cent for early phase and 8.7 per cent for later phase samples from symptomatic flock and none from asymptomatic flock. Whereas, ELISA detected that eight per cent of the samples at the first analysis while the percentage increased to 87 after three months. They opined that frequent PCR positive reactions in early phase of the infection was due to the shedding of bacteria in the milk and the corresponding serum samples were generally ELISA negative while lower PCR positivity rate and a high sero-prevalence rate in later weeks and after antibiotic treatment of the animals.

Conclusion

Listeriosis is one of the most abortifacient animal pathogens of zoonotic impact, affecting both public and animal health, with extensive animal's economic losses due to abortion and new born mortalities which reflects the need for controlling reproductive drawbacks in ruminants. Keeping this in view, the LLO based indirect ELISA designed in the present study had successfully measured the antilisterial antibodies in cattle and sheep sera in conjunction with its non-hazardous antigen, simplicity and rapidity indicate

that this assay could be used to screen large number of serum samples. Further, the test offers the advantage of using purified antigens which can be readily stored and transported. It does not require the maintenance of constant supply of fresh listerial cultures of numerous serotypes.

The use of LLO in immunoassays obviates the necessity of using multiple serotype antigens thus its application might be useful not only for the serodiagnosis of listeriosis, especially when bacteria cannot be isolated but also for epidemiological surveys. Further, by changing the species specificity of enzyme – conjugated antibody, the same procedure could be applied to other species of animals to detect antilisterial antibodies.

SUMMARY

VI. SUMMARY

1. A total of 235 cattle and 252 sheep sera samples, 80 Blood samples (40 each from cattle and sheep), 20 vaginal swabs (10 each from cattle and sheep) and 40 milk samples from mastitis cows were collected from animals with various reproductive disorders or history of abortion and apparently healthy animals for serology and isolation respectively with 40 each of cattle and sheep samples commonly evaluated for PCR and isolation.

2. Of 140 samples from cattle (90) and sheep (50) only one each of 40 blood samples from cattle and sheep were confirmed as *L. monocytogenes.* None of the mastitis milk samples and vaginal swabs yielded *L. monocytogenes* on culturing on to selective enrichment in UVM and PALCAM.

3. *L. monocytogenes* (1/2a) was selected for the LLO extraction using DEAE cellulose ion exchange chromatography. The method was found to be satisfactory with a protein yield of 740µg per ml.

4. The species – specific indirect ELISA was standardized for the detection anti-listerial antibodies in cattle and sheep. An antigen concentration of 190 ng per well, a serum dilution of 1:50 and 1:10,000 dilution of protein G HRPO conjugate were found optimum.

5. 18.3 per cent (44 out of 235) of cattle and 23 per cent (59 out of 252) of sheep sera samples tested were found positive by indirect ELISA.

6. One of 40 blood samples from cattle and two of 40 sheep blood samples from the state of Maharashtra found positive for PCR assay targeting hlyA gene of *L. monocytogenes*.

7. LLO based indirect ELISA was further evaluated against isolation and PCR for 40 common blood samples each of from cattle and sheep. Listeriolysin O-based indirect-ELISA did not agree with the cultural isolation or PCR. Only one of nine cattle seropositive for ALLO was positive by both isolation and PCR, whereas remaining eight (20.5%) seropositive for ALLO were culture and PCR negative. The rest of the 31 (79.4%) of 40 cattle samples seronegative for ALLO were culture and PCR negative as well. Similarly of 12 sheep samples seropositive for ALLO, only one was positive by bacterial isolation where as two were PCR positive. While, remaining 28 (71.7%) of 40 samples compared, were ALLO as well as isolation and PCR negative.

8. The LLO based indirect ELISA was standardized for the detection of antilisterial antibodies in cattle and sheep. The indirect ELISA is found to be a simple and economical test; it lends itself for automation and can be used as a preliminary screening test for a large number of sera samples.

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ABSTRACT

VIII. ABSTRACT

Conventional diagnostic techniques for the detection of *Listeria monocytogenes* are time consuming labour-intensive and lacks specificity and sensitivity. Listeriolysin O (LLO), an extra-cellular 58 kDa haemolysin, is produced by all the pathogenic strains of *L. monocytogenes* and reported to serve as a reliable indicator of infection in animals. In the present study, *L. monocytogenes* 1/2a reference strain was selected for LLO extraction from cell free BHI broth culture supernatant of reference strain and the crude extract was purified by DiEthyl Amino Ethyl Cellulose (DEAE-C) ion exchange chromatography for using as the candidate of an antigen in the development and standardization of indirect ELISA for the serodiagnosis of listeriosis in cattle and sheep and test was evaluated with PCR and isolation. Out of 235 cattle and 252 sheep sera samples screened by indirect ELISA, antibodies to LLO were detected in 18.3 per cent (44) and 23 per cent (59) of cattle and sheep respectively. Further, 40 each of the blood samples from cattle and sheep were processed for isolation and PCR assay targeting *hly*A gene and respective sera from same animals were included for LLO ELISA. Only one of nine cattle seropositive for ALLO was positive by both isolation and PCR, whereas remaining eight (20.5%) seropositive for ALLO were culture and PCR negative. The rest of the 31 (79.4%) of 40 cattle samples seronegative for ALLO were culture and PCR negative as well. Similarly of 12 sheep samples seropositive for ALLO, only one was positive by bacterial isolation whereas two were PCR positive. While, remaining 28 (71.7%) of 40 samples compared, were ALLO as well as isolation and PCR negative. Despite such discrepancies in findings, LLO based ELISA would be very helpful for diagnosing the Listeriosis at herd level and identifying infected animals for elimination from the herd.

Keywords: *Listeria monocytogenes*; Listeriolysin O; indirect- ELISA; PCR; *hly*A gene.