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Comparative efficacy of Internalin C-based peptide and listeriolysin O-based enzyme linked immunosorbent assays for serodiagnosis of listeric infection in goats

Samir Das[1]*, S V S Malik[1], Sameer Shrivastava[2], Pradeep Gandhale[3], Satish Kumar[2], Shabu Shoukat[1], Durga P Das[1], S B Barbuddhe[4] and D. B. Rawool[1]

[1]Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, 243122, India.
[2]Division of Animal Biotechnology, Indian Veterinary Research Institute, Izatnagar, 243122 India.
[3]Division of Virology, Indian Veterinary Research Institute, Mukteshwar Campus, India.
[4]ICAR Research Complex for Goa, Old Goa, Goa 403402 India.

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This is the first study ever carried out to develop and evaluate internalin C (InlC)-based serological assay for diagnosis of *Listeria monocytogenes* (LM) infection in goats using synthetic peptide as an antigen. Nine peptides representing major antigenic domains of InlC, a novel protein linked to the virulence of LM, were identified, analyzed, synthesized and employed in indirect enzyme-linked immunosorbent assay (ELISA) to evaluate their diagnostic potential using sera of goats experimentally inoculated with live and killed LM and from apparently healthy goats. Sera were screened by standardized indirect ELISA to reveal the antibodies against InlC (AInlC) as well as listeriolysin O (ALLO). Overall, the result revealed that the AInlC titres are lower than the ALLO titres. However, a fair correlation was observed between the titres of AInlC and ALLO in experimentally infected as well as apparently healthy goats. Based on the results obtained by both the ELISAs, it is suggested that InlC peptides alone may not serve as a suitable diagnostic antigen in indirect based ELISA for serodiagnosis of listeric infections. Further, there is need for identification, synthesis and evaluation of appropriate synthetic peptide(s) for essential virulence markers of listeriae whether existing or new marker, all need to be explored for developing an ultimate sensitive and specific rapid sero-diagnostics marker against listeriosis.

Key words: *Listeria monocytogenes,* Internalin C, Listeriolysin O, Peptide and enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Listeriosis is a bacterial disease characterized by neural, visceral and reproductive clinical entities, usually manifested as septicaemia, meningitis, encephalitis and abortion in humans and animals. It is mainly transmitted by ingestion of contaminated food and the disease is particularly common in ruminants fed on silage (Wagner and McLauchlin, 2008). It has emerged as an important food borne disease in human beings, especially in the developed world. Out of the eight known species of the *Listeria*, including the two new species *Listeria marthii* (Graves et al., 2010) and *Listeria rocourtiae* (Leclercq et al., 2010); *Listeria monocytogenes* and *Listeria ivanovii* are the only two pathogenic strains responsible for the illness. *L. monocytogenes* is responsible for 85% of animal cases and about 98% of human cases (Liu, 2006) with a very high mortality rate (20 to 30%) and ability to cause severe disease among the pregnant women, neonates and immune-compromised individuals (Esteban et al., 2009).

Many serological and molecular tests have been attempted

*Corresponding author. E-mail: drsamirvph@yahoo.com.

since the discovery of the bacterium with the aim of identifying the bacteria quickly, and with a greater degree of sensitivity (Jadhav et al., 2012; Shoukat et al., 2013). The humoral immune response is normally detected during listerial infection even in the absence of clinical symptoms. Many serodiagnostic assays employing the somatic (O), flagellar (H), cold-extracted and sonicated antigens of *Listeria* spp. have been developed for screening the animal and human listeriosis cases. However, these conventional assays cannot be relied upon for their poor specificity and sensitivity (Berche et al., 1990). Outer membrane protein (OMP) of *Listeria* spp. has also been used for developing Genus-specific as well as *L. monocytogenes*-specific enzyme-linked immunosorbent assays (ELISA) (Chen and Chang, 1996). However, these assays fail to discriminate between pathogenic and non-pathogenic *Listeria* strains. A number of virulence markers of *Listeria* species capable of eliciting the antibody response during listerial infection such as listeriolysin-O (LLO) (Berche et al.,1990); internalins (InlA, InlB, InlC, InlC2, InlJ etc.), the leucine rich repeat (LRR) proteins of *Listeria* spp. produced by virulence-linked family of genes (Boerlin et al.,2003); Act A protein associated with cell-to-cell spread of the agent (Ellin Doyle, 2001), two phospholipases C namely the PI-PLC encoded by *plc*A gene and the PC-PLC encoded by *plc*B (Chaudhari et al., 2004a,b); and the autolysin p60 protein (Hess et al., 1996). Out of the mentioned markers, LLO a 58-kDa secreted protein is one of the most important virulence factor produced by *L. monocytogenes* which can be detected soon after clinical onset of listeriosis and antibodies persist for at least several months (Berche et al., 1990; Shoukat et al., 2013). LLO based ELISA had been used (Low and Donachie, 1991; Low et al., 1992; Boerlin, 2003) however, LLO cross reacts with streptolysin, perfrinolysisn, suilysin and hence need prior adsorption at least with streptolysin O (SLO) before performing LLO-based ELISA (Berche et al., 1990). Hence, other virulence marker are being searched extensively to remove this pothole and in this regard, Internalin group of protein had proved potential especially InlC - being an immunodominant and virulence-linked protein, had shown promising result as diagnostic antigen in ELISA for screening the human clinical cases (Grenningloh et al., 1997) and experimental listeric infection in ewes (Zundel et al., 2007), where it proved superior to LLO-based ELISA for reliable diagnosis of listeric infection. It is noteworthy that barring the two non-pathogenic serovars of *L .monocytogenes* namely 4a and 4c (Liu et al., 2007), the InlC has been reported to be secreted by the pathogenic strains of all the serovars of *L. monocytogenes* including the most important serovars; that is, ½ a, ½ b and 4b; as well as the only serovar of *L. ivanovii*; that is, 5. Therefore, it would be rational to study the diagnostic potential of this important and novel virulence marker as a diagnostic antigen in a serological assay for reliable diagnosis of listeric infection by the pathogenic species of Listeria in man and domestic animals, particularly the goats, which are one of the most susceptible species. In view of the above facts, the present study was undertaken to develop and evaluate an ELISA employing synthetic peptides of InlC, for rapid and reliable serodiagnosis of listeric infection using sera of goats experimentally infected with live and killed LM as well as serum samples collected of apparently healthy goats. The developed InlC based indirect ELISA was compared further with LLO based indirect ELISA using the same sera samples to reveal it diagnostic sensitivity and specificity.

MATERIALS AND METHODS

Reference strains

The standard strain of *L. monocytogenes* 4b (MTCC 1143) was procured from IMTECH, Chandigarh. The strain was tested for its purity by morphological and biochemical characterization. The pathogenic potential was tested by *in vivo* pathogenicity tests in mice and the strain was passaged twice in mice for revival of its pathogenicity. The strain was maintained in the laboratory by monthly sub-culturing in brain heart infusion (BHI) broth.

Animals

The animal experiments were carried out after approval from Institutional Animal Ethics Committee. In brief eight healthy male Black Bengal goats (12 to 18 months old) were procured from the Livestock Production Research Section, Indian Veterinary Research Institute, Izatnagar, India. Prior to inclusion of these animals in the study, absence of listeric infection was ascertained by blood culturing as well as testing the serum of animal by Indirect based LLO ELISA. During the entire course of the experiment, the animals were fed *ad libitum* with fodder, concentrate and water. The goats were divided into three major groups, having five goats in group I, one goat in group II and two goats in group III, respectively. All the five goats of group I were orally infected each with 3×10^9 live cells of pathogenic *L. monocytogenes* MTCC, while the goat in group II was subcutaneously inoculated with 3×10^9 killed cells of pathogenic *L. monocytogenes* MTCC 1143. The two goats of group III, were kept as control of which one was given oral sterile PBS, whereas, another goat was subcutaneously inoculated with sterile PBS. The animals were kept under routine clinical observation and their rectal temperatures were recorded daily for 14 days post infection (PI). Besides, the presence of *L. monocytogenes* was determined in samples of blood and swabs from the rectum and nasal cavity, which were collected from all the experimental (infected and non-infected) animals till the end of the study; that is, day 60 PI. The bacteriological samples were processed immediately to isolate the pathogen whereas serum samples were stored at -20°C until tested by ELISA. Use of experimental animals complied with the guidelines of the Committee for the Purpose and Supervision of Experiments on Animals (The Gazette of India).

Indirect LLO enzyme-linked immunosorbent assay (ELISA)

Indirect based LLO ELISA was performed using purified LLO available at Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar. The purity of LLO was checked with SDS-PAGE while protein concentration was estimated using Bradford assay. Cross reactivity of LLO with SLO was avoided by

Table 1. Peptide sequences for Internalin C.

Code of candidate peptide	Sequence of peptide	Orientation	Position of amino acid in InlC sequence	Length
InlC.1M	TGSGTKVQAESIQRPTPINQ	MAP	25 - 44	20
InlC.2M	GVQNFNGDNSNIKSLA	MAP	75 - 90	16
InlC.3L	ELSMNRNRLKNLNGIPSAC	Linear	122 - 140	19
InlC.4L	VDNNELRDTDSL	Linear	146 - 157	12
InlC.5L	LSIRNNKLKSIVM	Linear	166 - 178	13
InlC.6L	HGNEITNTGGLTRLKKVNWI	Linear	191 - 210	20
InlC.7L	DLTGQKCVNEPVRYQPE	Linear	211 - 227	17
InlC.8L	ELYITNTVKDPDGRWIS	Linear	227 - 243	17
InlC.9L	DLTGQKCVNEPVRYQPELYI TNTVKDPDGRWI	Linear	211 - 242	32

adsorption of goat sera with SLO as per the method described by Berche et al. (1990) with certain modifications. These SLO-adsorbed goat sera were then used in the indirect plate ELISA to screen for ALLO as per the method described by Low et al. (1992).

Prediction and synthesis of InlC peptides

The protein sequences of *L. monocytogenes* and *L. ivanovii* for Internalin C gene were obtained from NCBI database. Prediction of peptides corresponding to antigenic epitopes of InlC was performed using bioinformatics software such as Protean-DNASTAR followed by basic local alignment search tool (BLAST) analysis of all individual peptides to confirm their individual specificity. In brief, analysis of the sequences of InlC protein, by Megalign revealed more than 91% homology between *L. monocytogenes* and *L. ivanovii*. The BLAST analysis proved conserved nature of this protein in both the pathogenic species. Nine short peptides (12-32 amino acids) were tentatively selected as 'probable candidate peptides' based on their hydrophobicity, antigenecity and surface probability with turn regions in 'Protein antigenecity plot' performed by 'PROTEAN' program of DNA-STAR vis-à-vis high score of these nine peptides obtained by on-line analysis from National Institute of Health (NIH), USA. The top scores of BLAST analysis of these nine 'probable candidate' peptides showed very high specificity for InlC of *L. monocytogenes* (~100 to 66%) and of *L. ivanovii* (~100%), and therefore, these were finally selected as 'probable candidate peptides'. Of these nine 'probable candidate' peptides, using solid phase chemistry, two peptides; that is, InlC.1M and InlC.2M were synthesized on alanine MAP (multi antigenic peptide) core, while rest of the other linear peptides; that is, InlC.3L, InlC.4L, InlC.5L, InlC.6L, InlC.7L, InlC.8L and InlC.9L were synthesized by Wang resin method (Table 1).

Standardization of indirect enzyme-linked immunosorbent assay (ELISA) for InlC peptides

InlC peptide based Indirect ELISA was standardized employing checkerboard titration method. In brief, the plates were coated overnight at 4°C with different peptides (100 µl/well) having different concentrations (10000, 5000, 2500, 1250 and 625 ng/well). Later, the plates were incubated at 37°C for one hour and washed thrice with phosphate buffer sulphate with Tween (PBST). Unsaturated sites were blocked with 200 µl of 0.5% BSA fraction V in PBS and incubated at 37°C for 2 h in humidified chamber. Later the plates were washed thrice by PBST.

Test sera were diluted serially from 1:25 to 1:32000 in blocking buffer which was then added to the plates at 100 µl/well. Both positive and negative sera were included in the assay. The plates were incubated at 37°C for 2 h and then washed thrice with PBST. Anti- goat IgG HRP conjugate (Sigma) at a dilution of 1:10,000 was then added (100 µl/well) and further incubated at 37°C for 1 h. After incubation, the plate was washed thrice by PBST. The reaction was visualized by adding substrate solution at 100 µl/well having 6 mg OPD (sigma) in 10 ml of citrate buffer and 5 µl of 30% hydrogen peroxide. The plates were then incubated at room temperature for 15 min in dark. Finally the reaction was stopped by adding 50 µl of 2N H_2SO_4 stop solution and read at 492 nm using ELISA reader (EC, India).

A serum sample at the dilution of 1:100 with the positive-to negative (P/N) ratio of ≥ 2 was considered positive for anti-InlC peptide antibodies in standardized ELISA and p/n ≥ 1.8 to < 2 was considered as doubtful.

Serum samples

A total of 121 serum samples collected from apparently healthy slaughtered goats as well as sera from experimental goats were screened for antibodies against LLO and InlC peptides using standardized indirect ELISA described above.

RESULTS

In the present investigation, goats experimentally inoculated with live as well as heat - killed pathogenic strain of *L. monocytogenes* by oral and subcutaneous routes, respectively, were screened initially for rise in body temperature followed by clinical signs if any. Later the sera of these animals were studied to observe kinetics of antibody production against listeriolysin-O (LLO) and InlC peptides using standardized indirect ELISAs.

Out of five goats inoculated with pathogenic strain of *L. monocyotgenes*, three showed a sharp rise in body temperature (upto 104 to 105°F) within one to two days of infection which lasted for about three to five days and then gradually declined to normal by day eight post-infection (PI). However, one goat showed slight rise (102 to 102.5°F) in body temperature, whereas the fifth goat did not reveal any sign of rise in temperature. None of the infected goats revealed typical circling movement suggestive of listeriosis.

Isolation of the pathogen from five inoculated goats

Table 2. Shedding pattern (Faecal/nasal) of LM in of experimentally infected goats.

Goat number	Type of culture/route	Post-inoculation (day)						
		0	3	7	10	14	21	28
(G1/LM/V)	LM Live Oral	-	+	+	-	-	-	-
(G2/LM/V)	LM Live Oral	-	+	+	-	-	-	-
(G3/LM/V)	LM Live Oral	-	+	+	-	-	-	-
(G4/LM/V)	LM Live Oral	-	ND	ND	ND	ND	ND	ND
(G5/LM/V)	LM Live Oral	-	-	-	-	-	-	-
(G1/LM/K)	LM killed S/C	-	-	-	-	-	-	-
C1	Nil oral	-	-	-	-	-	-	-
C2	Nil s/c	-	-	-	-	-	-	-

ND - Not done

was attempted from their blood, rectal and nasal swabs taken at different intervals during the course (60 days) of the study (Table 2). The listeriae could not be isolated from blood in any of the infected goats, however, nasal and fecal swabs, revealed cultural positivity in case of three inoculated animals on day three and seven PI (Table 2). One of the infected goats suffered from diarrhea and died on day 60 PI. Pathological examination of the goat revealed enteritis and encephalitis; and the brain tissue smear showed Gram positive rods which were further confirmed by isolation and biochemical characterization. However, owing to liquefactive changes in brain, the presence of micro-abscesses could not be appreciated. The goat inoculated subcutaneously with killed culture of pathogenic *L. moncytogenes*, did not show any rise in the body temperature nor revealed the presence of the pathogen in the faecal and nasal sheddings during the study period (Table 2). Both goats kept as uninfected controls also had no signs of rise in the body temperature and did not reveal the presence of the pathogen in their sheddings (Table 2).

Kinetics of antibody production against listeriolysin-O (ALLO)

Prior to inclusion of goats in the study, all the goats intended for use in experimental study were tested for absence of antibodies against LLO (ALLO) using standardized indirect plate ELISA. Post infection, an appreciable sero-conversion against LLO was observed in four of five goats inoculated with *L. monocytogenes* with a positive to negative (p/n) ratio of ≥ 2 from day 14 onwards, which increased thereafter. The titer of the (ALLO) peaked during 21 to 28 days PI and then decreased gradually. ALLO titers remained detectable till the end of the experiment (upto 60 days) (Figure 1). However, ALLO could not be detected in the goat inoculated with heat killed culture of *L. monocytogenes* and also in uninfected control goats (Figure 1).

Kinetics of antibody production against Internalin-C (AInlC)

Before analyzing the kinetics of antibody production against InlC peptides, the nine 'probable candidate' synthetic peptides (InlC.1M to InlC.9L) were screened initially with sera of experimentally infected goats by standardized indirect ELISA. Of the nine InlC peptides, three peptides namely, InlC.1M, InlC.3L and InlC.7L showed significant sero-positivity, and therefore, finally named as 'candidate peptides'. Further, combinations of candidate peptides were made and referred to as "Mixotopes"- Mx1(InlC.1M and InlC.3L), Mx2 (InlC.1M and InlC.7L), Mx3 (InlC.3L and InlC.7L) and Mx4 (InlC.1M, InlC.3L and InlC.7L). These mixotopes combinations were screened by indirect ELISA using the sera collected from experimental animals. On analysis of results, it revealed that the Mx4 was found to be the best mixotope, amongst all tested individual peptides and their combination and its BLAST analysis showed specificity to InlC of *L. monocytogenes* and *L. ivanovii*.

On day zero; that is, the day on which goats were experimentally inoculated, the sera of all experimental goats (eight) were found to be negative for antibodies against InlC (AInlC) when tested by peptide (Mx4)-based indirect ELISA. Later, an appreciable sero-conversion was observed against InlC with a p/n ratio ≥ 2.0 in three out of five *L. monocytogenes* inoculated goats on day 14 PI, which increased thereafter, and attained peak by days 21 to 28 PI by peptide-based ELISA. Subsequently, the titres decreased gradually but remained detectable at non-significant levels upto day 40 PI (Figure 2). AInlC titer was not elicited in the goat inoculated subcutaneously with heat killed culture as well as in the goats kept as control (Figure 2).

Comparison between the ALLO-and AInlC- positivity

Antibodies against LLO (ALLO) and InlC (AInlC) in sera of goats inoculated with live *L. monocytogenes* were detected by indirect ELISA on day 10 to 14 PI, with peak titres on day 21 to 28 PI. Subsequently, the ALLO and AInlC titres showed a gradual decline and were non-significant on day 60 PI and day 40 PI, respectively (Figures 1 and 2). Significant titres of ALLO were observed in four out of five animals inoculated with live *L. monocytogenes* whereas, only three of these animals showed significant antibody titres against InlC. The goat inoculated with heat

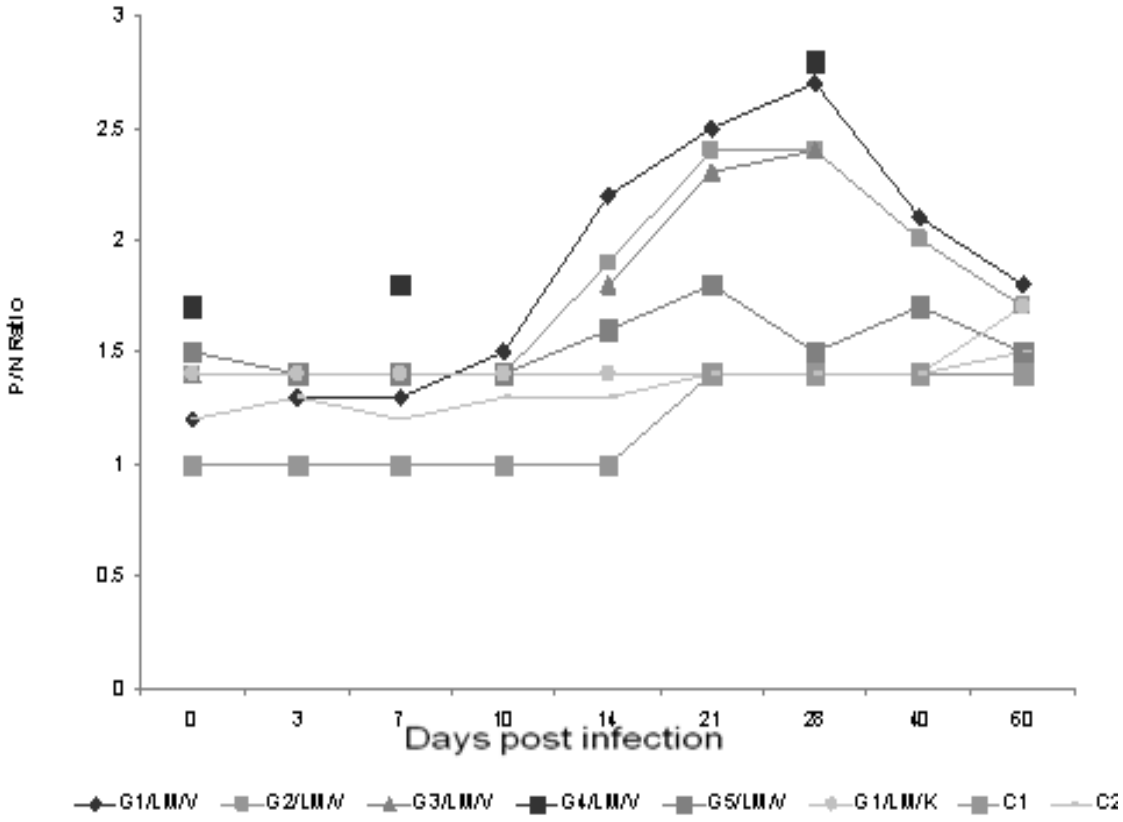

Figure 1. Kinetics of LLO antibodies in experimental goats by indirect ELISA. G: goat, LM: Listeria monocytogenes, V: viable/live, K: killed, C: control goats.

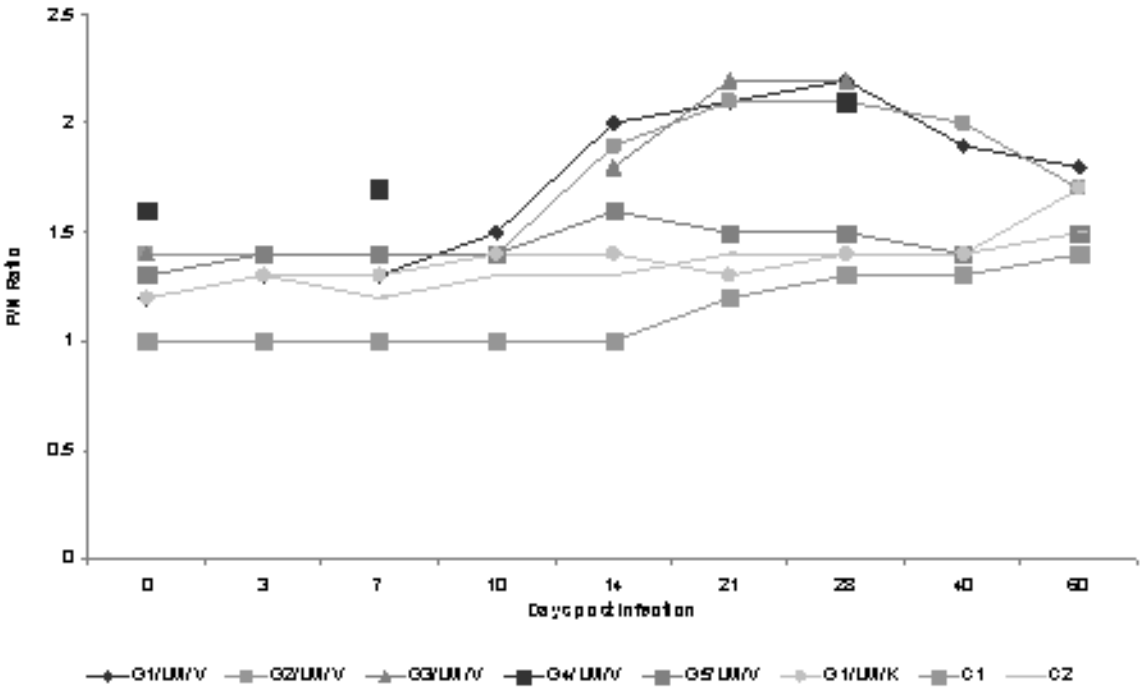

Figure 2. Kinetics of InlC peptide antibodies in experimental goats by indirect ELISA. G: goat, LM: Listeria monocytogenes, V: viable/live, K: killed, C: control goats.

Table 3. Efficacy of different ELISA's in apparently healthy goats (n=121).

Status	Criterion	No. positive	Percent positive
LLO+ve	p/n>2	23	19
LLO +ve (SLO adsorbed)	p/n>2	13	10.7
InlC Mixotope +ve	p/n>1.8	25	25.6
InlC Mixotope +ve	p/n>2	6	4.9
LLO +ve (SLO adsorbed) plus InlC Mixotope +ve	p/n>2	4	3.3

killed organisms as well as uninfected goats kept as negative controls did not reveal ALLO or AInlC titers in their sera (Figures 1 and 2).

Screening of field sera

On screening of sera from apparently healthy slaughtered goats (121) by the InlC-based ELISA (p/n ratio ≥ 1.8), InlC ELISA (p/n≥2), LLO-based ELISA (p/n≥2) and LLO-based ELISA (after adsorbption of sera with streptolysin) (p/n ≥ 2) revealed the seropositivity in 25.6, 4.9, 19 and 10.7% goats, respectively (Table 3). On comparison, an overall seropositivity of 3.3% was observed in indirect ELISA against InlC-(p/n ≥ 2) and LLO (After adsorption of sera with SLO). Although, InlC-based ELISA could detect seropositivity in two more cases, however, seven serum samples which showed positivity in LLO based ELISA (after SLO adsorption) were found negative in InlC-based ELISA.

DISCUSSION

To elucidate the role of antibodies in protection against listerial infection, studies aiming to identify and characterize new antibody targets are necessary. Under this context a study based on sequence analysis and western blot analysis identified eight *L. monocytogenes* proteins, three internalin members (InlA, InlD and InlC2) and five novel proteins of unknown function (designated IspA, IspB, IspC, IspD and IspE, respectively) as targets of humoral immune responses during listerial infection. These proteins were found to induce humoral immune responses during infection and thus opinioned to be useful candidates for serodiagnosis, vaccine and drug development (Yu et al., 2007). The candidate antigen, the InlC when employed as diagnostic antigen in recombinant InlC (rInlC) based ELISA gave promising results in detecting serological responses in experimental infected ewes (Zundel et al., 2007) whereas purified InlC earlier used in immunoblot have shown variable results in clinical cases of human Listeriosis (Grenningloh et al.,1997). Therefore in the present study we decided to use this protein in development of indirect based ELISA for serodiagnosis of listeric infections utilizing synthetic peptide based approach.

Oral dosing of pathogenic strain of *L. monocytogenes* caused no illness in goats except for mild pyrexia and diarrhea. In the present study, the recovery of *L. monocytogenes* from the brain of one goat suggested characteristic listeric encephalitis, which is in agreement with reported association of this pathogen with cases of encephalitis in goats (Yousif et al*.,* 1984; Oevermann et al*.,* 2010). In our study two goats failed to reveal an appreciable increase in body temperature and the presence of the pathogen in its faecal and nasal swabs. These intra-species variations might be due to the differences in the humoral and cellular responses of the experimentally infected animals against *L. monocytogenes* (Miettinen et al*.*, 1990; Bhanurekha et al*.,* 2006). Besides, killed culture of *Listeria* failed to elicit any clinical response (Yu et al*.,* 2007) which was concurrent with the results obtained in the present study. LLO, being a secretary protein which secreted only by live bacterial cells, therefore none of the goats inoculated subcutaneously with killed culture of pathogenic *L. monocytogenes* showed rise in temperature, pathogen isolation and ALLO, which is in accordance with similar observations reported in case of rabbits infected with *L. monocytogenes* (Yu et al*.*, 2007).

An appreciable ALLO was detected on day 14 PI, with peak between 21 to 28 days in case of four out of five *L. monocytogenes* inoculated goats and titres were detectable till 60 days PI in our study was in concordance with similar studies of (Bhanu Rekha et al., 2006; Miettinen et al., 1990).

In the present study, a significant sero-conversion with high levels of antibodies against InlC (AInlC) was observed in three out of five *L. monocytogenes* infected goats on day 14 PI which peaked on days 21 to 28 PI. These observations suggest an active listeric infection in these goats which showed initial appreciable rise in their body temperature and also shedding of the pathogen in their faecal/nasal samples till day 7 PI. Failure of the recovery of pathogen and absence of AInlC noticed in one *L. monocytogenes* - inoculated goat might be attributed to the intra-species variations in the immunological response or even complete absence of any serological response in animals given low infective doses, like that witnessed as sero-negativity of all the ewes in rLLO- and InlA-based ELISAs following their experimental infection with low doses (10^4 to 10^6 live organisms) of *L. monocytogenes* (Zundel et al*.*, 2007). Another possibility could be due to lower specificity or sensitivity of InlC peptide-based ELISA employed in our study, which might have failed to detect AInlC in this goat.

Unlike our study, wherein detectable titre of ALLO persisted for longer period (upto day 60 PI) in comparison to that of AInlC (upto day 40 PI) in goats experimentally infected with live *L. monocytogenes*, both type of antibodies (ALLO and AIrpA) remained detectable up to the end of study (day 48 PI) in ewes experimentally infected with *L. monocytogenes* (Zundel et al*.,* 2007). In terms of their efficacy, both the ELISAs (LLO-and InlC peptide-based) employed in our study were found to be specific for detecting the true negative animals; that is, animals inoculated with killed *L. monocytogenes* as well as uninfected controls.

It has been reported that the internalin A-related protein IrpA, also called internalin C is associated with the virulence of *L. monocytogenes*, and is a major protein target of the human humoral response to *L. monocytogenes* (Grenningloh et al., 1997). However, on comparison of SLO-adsorbed LLO-based ELISA (p/n ≥ 2) and InlC-based ELISA (p/n ≥ 2), respectively, an overall seropositivity (3.3%) was quite low. Besides, the latter test detected two more sera as positive for AInlC that were found negative for ALLO (SLO adsorbed sera). In contrast to these findings seven sera that were found positive for ALLO (SLO adsorbed sera), turned out to be negative for AInlC. These findings suggest that InlC-based peptide ELISA requires further improvement in order to make it more specific and sensitive through the use of mixotope(s) of InlC peptide(s) with other peptide(s) directed against more reliable virulence markers of listeric infection.

Results of experimental and spontaneous studies on listeric infection in goats reveal that antibodies against InlC protein and against LLO were elicited in goats following their experimental infection, and these were also detected in sera of apparently healthy goats by InlC peptide-based and LLO-based ELISAs, respectively. However, in the absence of an appreciable correlation between AInlC and ALLO, further improvement in ELISA is needed, particularly in terms of identification, synthesis and evaluation of appropriate synthetic peptide(s) for essential virulence markers of listeriae. These observations although are in contrast with that of an earlier report in ewes experimentally infected with *LM*, wherein rIrpA-based ELISA was claimed to show more promising results than LLO-based ELISA (Zundel et al., 2007), but at the same time, both the approaches were not identical for ideal comparison.

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