RESEARCH ARTICLE

Listeriolysin O based Serodiagnosis *Vis-A-Vis* Cultural Isolation of *Listeria monocytogenes* from Slaughtered Goats

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Abstract

The present study was undertaken to ascertain prevalence, pathogenicity of listeriosis *vis-a-vis* seroprevalence of *L. monocytogenes* among goats slaughtered at Nagpur. The organism was isolated, identified, and characterized following USDA protocol from a total of 202 samples comprising blood (101) and chevon (101) samples revealing 0.99% prevalence of pathogenic *L. monocytogenes*. The virulent nature of pathogenic *Listeria* isolates was also assessed by *in-vitro* tests like haemolysis on sheep blood agar, CAMP test and PI-PLC assay. Listeriolysin-O (LLO) protein produced by *L. monocytogenes* was purified employing ion-exchange chromatography. The purity of the LLO was confirmed using SDS-PAGE, which revealed it to be a homogeneous 58 kDa protein. Hyper immune serum was raised against purified LLO in healthy rabbits. An ELISA was standardised by the checker-board titration method and using LLO as an antigen and anti-LLO antibodies in raised hyper-immune serum. The LLO-based indirect ELISA screening of streptolysin-O adsorbed sera from slaughtered goats demonstrated seropositivity for ALLO in 13.86% samples. Moreover, a positive correlation between seropositivity and isolation of pathogenic *L. monocytogenes* from the same animal was also noted endorsing the active functioning of ALLO in infected animals. The results advocate use of LLO-based indirect ELISA on SLO adsorbed sera for rapid and reliable serodiagnosis of listeriosis in large populations, where traditional isolation methods are not feasible.

Key words: ELISA, Goats, *Listeria monocytogenes*, Listeriolysin O, SDS-PAGE.

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Introduction

Listeriosis is a serious foodborne bacterial zoonosis caused by pathogenic strains of two *Listeria* species, *Listeria monocytogenes* and *Listeria ivanovii*. *L. monocytogenes* is an important foodborne pathogen not because it causes a large number of symptomatic cases, but because of its relatively high case-fatality rate (Dhama *et al*., 2015). The genus *Listeria*, also contains other 24 non-pathogenic *Listeria* species (Barbuddhe *et al*., 2022). The disease listeriosis, which is classified as OIE List C, causes neural, visceral, and reproductive disorders in various species of animals as well as humans, who are immunocompromised or in contact with animals (Suryawanshi *et al*., 2014).

Isolation of the pathogen is the most reliable way to diagnose listeriosis. However, it takes 2-3 days to provide presumptive positive results and another 2-4 days to confirm suspected colonies using biochemical tests (Jadhav *et al*., 2012). Serological tests, on the other hand, have the advantage of a large number of mass screenings, as well as being inexpensive, simple to perform, and interpret. Ideally, such a test would have enough sensitivity and specificity to detect the humoral response directed against the agent's immunogenic components, preferably those linked to its virulence (Shoukat *et al*., 2013). Listeriolysin-O (LLO) expressed by *L. monocytogenes* strains has been widely used in ELISA for the diagnosis of listeric infection in humans (Barbuddhe *et al*., 1999; Suryawanshi *et al*., 2014), goats (Suryawanshi *et al*., 2017), sheep (Suryawanshi *et al*., 2017), cattle and buffaloes (Suryawanshi *et al*., 2017). The present investigation was carried out with the objective to assess the prevalence, *in-vitro* pathogenicity, and detection of listeriolysin-O (LLO) protein profiles of pathogenic *Listeria* species isolates from slaughtered goats at Nagpur city of Maharashtra.

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Materials and Methods

Ethical Approval

The research work was duly approved by Institutional Animal Ethics Committee (IAEC).

Sample Collection

In all, 202 samples were collected and screened for microbiological evaluation, including blood samples (n=101) and chevon samples (101) from corresponding goats with

either a history of abortion and/or apparently healthy animals from slaughterhouses in Nagpur, Maharashtra, India. Chevon samples were collected in a sterile zip lock bags, while blood samples were collected in sterile vials.

Bacterial Strains

The standard strains *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 3160) and *Rhodococcus equi* (MTCC 1135) used in this investigation were obtained from the Microbial Type Culture Collection Centre at the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Isolation and *In Vitro* Pathogenicity Test of *L. Monocytogenes*

The samples were collected aseptically and immediately processed for *Listeria* species isolation using the U.S. Department of Agriculture (USDA) method as described by Curtis and Lee (1995), with appropriate modifications, including two-step enrichment with the University of Vermont (UVM-I and II) and subsequent streaking onto polymyxin-Acriflavin-Lithium chloride Ceftazidime Aesculin-Mannitol (PALCAM) medium as a selective agar. The recovered isolates were phenotypically characterised using an array of biochemical and sugar fermentation tests. Gram's staining, tumbling motility at 20-25°C, catalase, oxidase, Methyl Red-Voges Proskauer (MR-VP), and nitrate reduction tests were performed in biochemical testing, while sugar fermentation tests were performed with rhamnose, xylose, mannitol and α-methyl-d-mannoside. The biochemically confirmed *L. monocytogenes* isolates were subjected to *in-vitro* pathogenicity assessments which included haemolysis on 7% sheep blood agar (SBA), CAMP (Christie, Atkins, Munch-Petersen) test and Phosphatidyl-inositol-Phospholipase C (PI-PLC) activity on L. mono Differential Agar medium (HiMedia Labs, India).

Antigen

Ion-exchange chromatography was used to prepare and purify culture filtrate antigen (CFA) from the cell free supernatant of 18 h old *L. monocytogenes* (MTCC 1143) bacterial growth in Brain heart infusion (BHI) broth (HiMedia Labs, India) (Lhopital *et al*., 1993). It was precipitated at 50% ammonium sulphate saturation and dialyzed overnight against phosphate buffered saline (PBS), pH 7.2. SDS-PAGE was used to confirm the purity of the LLO, which revealed it to be a homogeneous 58.0 kDa protein. The LLO-containing fractions were pooled, and the protein content was determined using the BCA™ Protein Assay kit (Pierce, USA, Catalog No. 23225) before being stored at -20°C until use.

Protein Profile Employing SDS-PAGE

Protein profiling was performed on all five *Listeria* spp. isolates using the Laemmli protocol (1970). In brief, all *Listeria* species isolates were inoculated in 20 mL BHI broth for 12 h with continuous aeration (shaker) at 37°C. The supernatants were collected by centrifuging the freshly grown cultures at 40°C at 12,750 g for 5 min (REMI, India). The culture supernatants were filtered through 0.45 nitrocellulose acetate filters to obtain cell free culture supernatants.

Raising Antisera against Purified Listeriolysin-O (LLO)

In two adult healthy rabbits, hyper immune serum was raised against purified LLO. 100 µg of purified LLO was dissolved in 1 mL of NSS and mixed with 1 mL of Freund's complete adjuvant (FCA) before being administered intravenously at multiple sites. The titre was determined by testing the rabbits 14 days after the injection. After adding Thiomersal @ 0.01% as a preservative, the anti-LLO serum (Hyperimmune serum) was separated and stored at - 20°C.

Indirect ELISA

The indirect plate ELISA was performed using the method described by Low *et al*. (1992). To standardise the ELISA, the checker-board titration method was used. In a standardised ELISA using LLO antigen (1 µg/well) and rabbit anti-goat HRPO conjugate (1:2000, Sigma-Aldrich, India, Product No. A8792), a serum sample at a dilution of 1:200 with a positive to negative (P/N) ratio of more than 2 was considered positive for listeriosis. This standardised ELISA tests was then used to screen for anti-LLO antibodies before and after test sera adsorption with Streptolysin-O (SLO) (Sigma-Aldrich, USA, Product No. S5265) according to the protocol described by Berche *et al*. (1990). All the test sera were evaluated three times independently by ELISA.

Results and Discussion

Prevalence of *Listeria* species in Slaughtered Goats

Microbiological analysis of 202 samples from slaughtered goats revealed presumptive *Listeria* species isolates in four chevon (G55, G48, G23, G54) and one blood (GB89) clot samples. Subsequently, on morphological and biochemical characterization, two *L. monocytogenes* isolates and three *L. seeligeri* isolates were identified. Both *L. monocytogenes* isolates (G55, G48) were isolated from chevon samples, whereas two of the three *L. seeligeri* isolates (G23, G54) were isolated from chevon and one from blood clot sample (GB89). The overall prevalence of *L. monocytogenes* and *L. seeligeri* from slaughtered goats was found to be 0.99% and 1.49%, respectively (Table 1).

Table 1: Prevalence of *Listeria* spp. in chevon and blood samples

		Listeriae positive		Species			
				L. monocytogenes		*L. seeligeri*	
Source	No. of samples	No.	%	No.	%	No.	%
Chevon	101	04	3.96	02	1.98	02	1.98
Blood	101	01	0.99	00	00	01	0.99
Overall	202	05	2.48	02	0.99	03	1.49

The low prevalence of *Listeria* spp. detected in chevon in present study is in agreement with the results stated by Singh *et al*. (2019) with a 1.82% (9/493) prevalence of *Listeria* spp. from retail meat shops and slaughter-houses of Punjab, India, wherein 1.62% (8/493) was *L. monocytogenes* and 0.2% was *L. seeligeri* (1/493). Similar levels of *L. monocytogenes* contamination of chevon samples were also reported from different locations of India like Punjab (Malik *et al*., 2012), Karnataka (Kumar *et al*., 2014) and Kerala (Latha *et al*., 2017). Yet several investigations have noted a significantly higher recovery of *Listeria monocytogenes* in chevon ranging from 6.66% to 25.5% (Barbuddhe *et al*., 2000; Shakuntala *et al*., 2019). These variable prevalence estimates may be explained by differences in sample quantity and origin, detection methods, and the hygienic status of meat markets and the personnel involved in slaughtering and handling meat at various levels.

In-vitro Pathogenicity Testing of *Listeria* Isolates:

The pathogenic potential of all five *Listeria* species isolates recovered in the current experiment was assessed using *in-vitro* techniques. The isolates were put through a series of *in-vitro* pathogenicity tests. On performing CAMP test, both the *L. monocytogenes* isolates showed enhanced zone from partial haemolysis to a wider zone of complete haemolysis towards *Staphylococcus aureus* (MTCC 3160), indicating their virulent nature. Similar results were also observed regarding PI-PLC assay. Within 24 h of incubation, both *L. monocytogenes* isolates showed prominent PI-PLC activity on L. mono Differential Agar medium, whereas none of the three *L. seeligeri* isolates displayed opaque halos around the colonies. Similar tools of *in-vitro* pathogenic assays were also reported to be used by Negi *et al*. (2016).

Protein Profile using SDS-PAGE

Except for a few major bands that appear to be shared by all *Listeria* species, SDS-extracted patterns in protein profiling are strictly species-specific (Tabouret *et al*., 1992). LLO is a crucial virulence marker of *L. monocytogenes* and a proven dominant antigen target of anti-listerial immunity (Bouwer *et al*., 1992) that stimulates T-cell recognition during an acute listeric infection. Only virulent strains of *L. monocytogenes* produce listeriolysin-O, a 58 kDa protein that has been widely employed as an antigen in the creation of the enzyme-linked immunosorbent test (ELISA) for the serodiagnosis of listeric infection (Barbuddhe *et al*., 2000; Suryawanshi *et al*., 2014). In current investigation, all *Listeria* isolates had at least six to twenty-five bands in their protein profiles using SDS-PAGE, with the lowest band measuring 9.63 kDa and the highest 160.26 kDa. The finding of protein bands in the 50–60 kDa range (57.328 kDa in G55 and 58.159 kDa in G48) (Table 2, Fig. 1) may represent haemolysin (LLO), which suggests a connection between the protein patterns and the virulence of isolates demonstrated by *in-vitro* studies.

Table 2: Details of Protein profiles of *Listeria* isolates

Isolate No.	**MTCC 1143**		**G55**	**G48**	**G23**	**G54**	**GB89**
Isolate	***L. monocytogenes***	**Standard marker**	***L. monocytogenes***	***L. monocytogenes***	***L. seeligeri***	***L. seeligeri***	***L. seeligeri***
Source	**Meat**		**Meat**	**Meat**	**Meat**	**Meat**	**Blood**
Mol. Wt (kDa)	142.330	97.400	145.910	160.264	159.422	142.052	134.794
	135.160	66.000	137.057	145.085	144.304	135.503	112.782
	71.204	43.000	98.155	135.536	136.216	118.232	68.891
	60.248	29.000	86.155	114.624	113.375	104.799	58.064
	53.041	20.100	80.506	68.251	88.146	97.381	46.011
	47.295	14.300	71.033	**58.159**	81.052	88.609	40.661
			67.422	51.780	67.461	85.865	38.508
			57.328	44.637	57.689	80.628 67.461	**30.015**
			50.737	39.554	43.976	57.318	
			45.193	37.784	38.508	44.837 42.812	
			42.481	**29.715**	**28.617**	41.485	
			40.473			38.508	
			37.370			35.338	
			36.424			**29.589**	
			32.594				
			28.771				
			28.018				
			25.739				
			23.521				

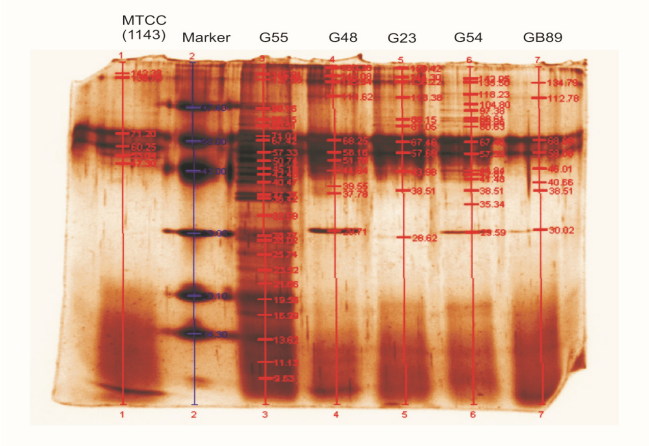

Fig. 1: Protein profile of listerial isolates using SDS–PAGE

Serodiagnosis by Indirect ELISA

The purified LLO-based ELISA is still the most accurate and reliable serodiagnostic assay available today for detecting listeric infection in both humans (Kaur *et al*., 2010; Suryawanshi *et al*., 2014) and animals (Shoukat *et al*., 2013; Suryawanshi *et al*., 2017). In the current investigation, indirect ELISA screening of sera from slaughtered goats using the antigen LLO demonstrated seropositivity for ALLO in 13.86% (14/101) samples (Table 3). The findings of the current study are consistent with those reported by Suryawanshi *et al*. (2017), who found that goats from the Nagpur region with a history of abortion, emaciation, and/or apparently healthy animals had a high seropositivity for ALLO (22.46%). Similar results were reported by Barbuddhe *et al*. (2000), who observed 33.8% and 41.1% seropositivity in apparently healthy sheep and goats when tested against pure LLO used as antigen in indirect plate ELISA.

Table 3: Seropositivity for anti-listeriolysin O antibodies by indirect ELISA

Cultural status	No. of sample	ALLO positive		ALLO negative	
		No.	%	No.	%
Isolation of *L. monocytogenes*	02	02	100	00	00
Isolation of *L. seeligeri*	03	00	00	03	100
Cultural negative	96	12	12.5	84	87.5
Overall	101	14	13.86	87	86.14

However, in the current study, LLO-based ELISA showed seropositivity for ALLO in animals that were culturally negative for *L. monocytogenes* and any non-pathogenic *Listeria* species (Table 3). Similar findings were also reported in earlier investigations, where ALLO antibodies were found in animals with negative cultural status (Barbuddhe *et al*., 2000; Suryawanshi *et al*., 2017). Since prior exposure and ALLO persistence in animals are expected long after the end of an antigenic stimulation, the high positivity for ALLO in the absence of clinical disease observed in our study could be attributed to the animals being exposed to low infective doses, which have been shown to elicit a persistent immune response to LLO equivalent to higher infective doses (Lhopital *et al*., 1993). Other haemolysins, including pneumolysin from *Streptococcus pneumoniae*, perfringolysin-O from *Clostridium perfringens*, cereolysin-O from *Bacillus cereus*, and alveolysin from Bacillus *alvei*, have also been reported to share common antigenic domains with LLO (Geoffroy *et al*., 1987). Hence, the cross reactivity that may result from these identical proteins expressed by other organisms can be the cause of the seropositivity seen in culturally negative animals in this study. Adsorption of the test sera with SLO has been found to cause 3-fold reduction in ALLO titers owing to excluding the noticeable cross-reactivity in animal listeriosis cases in cattle (Suryawanshi *et al*., 2017), sheep (Shoukat *et al*., 2013), goat (Suryawanshi *et al*., 2017) and human (Suryawanshi *et al*., 2014). The current study also found a strong correlation between ALLO positivity (seropositivity) and cultural isolation of pathogenic *L. monocytogenes* from the same animal (Table 4), confirming the function of ALLO in infection control. The findings are also consistent with previous reports on listeric infection seropositivity in animals (Lida *et al*., 1991) and humans (Aljicevic *et al*., 2006).

Table 4: Correlation between cultural positivity (isolation) and detection of ALLO

Isolation			ALLO Detection	
Sample No.	Source	+/-	Species identified	+/-
G55	Chevon	+	*L. monocytogenes*	+
G48	Chevon	+	*L. monocytogenes*	+
G23	Chevon	+	*L. seeligeri*	-
G54	Chevon	+	*L. seeligeri*	-
GB89	Blood	+	*L. seeligeri*	-

(+ Positive, - negative)

Conclusions

The present research findings revealed 1.98% prevalence of pathogenic *Listeria* species and 13.86% seropositivity for ALLO using an LLO-based ELISA in slaughtered goats in Nagpur, Maharashtra. Apart from the observation of positive correlation between seropositivity and isolation of pathogenic *L. monocytogenes* from the same animal, endorsing the role of ALLO in infection control, utility of protein profiling as method for determining pathogenicity was also established. Furthermore, LLO-based indirect ELISA on SLO adsorbed sera can be used for rapid and reliable serodiagnosis of listeriosis in large populations, where traditional isolation methods are not feasible.

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