

Original Research Article

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Isolation of *Listeria monocytogenes* and *Listeria ivanovii* and its Nitric Oxide Expression Level in Serum, Brain and Reproductive Organs of Sheep

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ABSTRACT

Two species of *Listeria* are pathogenic; *L. monocytogenes* infects humans and animals, and *L. ivanovii* infects ruminants only have been considered. While infection with *L. monocytogenes* and *L. ivanovii* are known as important causes of abortion and reproductive failure in ruminants and the role of nitric oxide (NO) expression in different organs of affected animals in India is limited. Therefore, the current study, (n= 78) serum and organs (comprising of 26 ovaries, 26 uterus and 26 brain) samples from the local abattoir in and around Jammu region were taken and evaluate for the presences of *Listeria* spp. by employing two fold enrichment procedure for isolation and confirmed by biochemical tests. Then further, pathogenicity of isolated were confirmed by Phosphatidylinositol-specific phospholipase C (PI-PLC) assay. Out of them *L. monocytogenes* was detected in 5 samples i.e. 3 in uterus, 1 in ovary and 1 in brain, and *L. ivanovii* was isolated only in 3 uterus sample with over-all prevalence of 6.41% and 3.85% respectively. The prevalence of other *Listeria* spp. in the sample was 3.85%. Further, we were found high concentration of NO in *Listeria* spp. infected samples in comparison to the *Listeria* spp. was not isolated. This study demonstrated that *Listeria* spp. (mainly *L. monocytogenes* and *L. ivanovii*) infected sheep showed very high NO concentration level compared to without infected animals. The functional role of nitric oxide (either iNOS or nNOS) needs to be further studied in natural and experimental models with *Listeria* spp. infection.

Keywords

Listeria ivanovii, *L. monocytogenes*, Sheep, Prevalence

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Introduction

Listeria spp. is a gram positive facultative intracellular and invasive bacterium. The genus *Listeria* contains 2 pathogenic

species, *L. monocytogenes* and *L. ivanovii* (Seeliger *et al.*, 1986; Lotfollahi *et al.*, 2017). *L. ivanovii* infection is associated with fetal death, stillbirths and premature births in ruminants, although it is less common

than *L. monocytogenes* (Low and Donachie, 1997). They both invade host cells, replicate in the cytosol after phagosomal escape, and spread from cell to cell by polymerizing actin. However, each species appears to infect different hosts: *L. monocytogenes* infects humans and ruminants, whereas *L. ivanovii* is thought to infect ruminants only (Vázquez-Boland *et al.*, 2001). It is an emerging foodborne zoonotic pathogen of high public health significance and is responsible for 60% of hospitalizations annual mortality of 20-40% (Low and Donachie, 1997). The ruminants are the major reservoir of *L. monocytogenes* in which infection is generally asymptomatic but could be manifested as encephalitis, abortion, septicemia and mastitis with considerable high economic losses (Low and Donachie, 1997).

It is known that NO can directly and indirectly modulate the immune response through diverse mechanisms within macrophages and other phagocytes. That is, it works as a microbiostatic or microbicidal agent or modulates biological activity of innate and acquired immunity cell lines (Heck *et al.*, 2005). Basic function of NO in immune system is its contribution in protective immunity against intracellular pathogens including viruses, bacteria and protozoa (Nathan 1991, Fang 1997, De Groote and Fang 1995 Singh *et al.*, 2018a,b). Its mechanism depends upon production of NO and superoxide (O₂⁻) by activated macrophages, which causes formation of peroxynitrite anion (ONOO⁻), which causes effect on pathogens. (Hess *et al.*, 2005; Sun *et al.*, 2006). Thus the knowledge of NO is not only important in understanding immunology but it is also a foundation for the development of new approaches for the management and treatment of various diseases, including *Listeria spp.* Based on above information, the aim of this study is to isolate and monitor NO expression level in serum, ovary, uterus

and brain of naturally affected slaughtered sheep with *Listeria. spp.* infection.

Materials and Methods

In the current study the (n=78) serum and organs (comprising of 26 ovaries, 26 brain and 26 uterus) samples from the local abattoir in and around Jammu were taken and evaluated for the presence of *Listeria spp.* in meat and the concentration of nitric oxide in the same samples were studied. Reproductive tracts along with the brain of same animals were collected and transported to the laboratory in Phosphate Buffered saline (PBS) in an ice box within 30 minutes. Hypothalamus was separated carefully from the brain, thoroughly rinsed in physiological saline and stored at -20°C till further use.

Follicular fluid was aspirated by using a separate hypodermic tuberculin syringe from each ovary. It was collected by applying slight pressure to avoid additional traumatization of follicles. Aspirated fluid was centrifuged at 3000 rpm for 15 minutes to remove cellular debris. The sample was kept at -20°C till further use. Samples of reproductive tissues (ovarian, oviductal and uterine tissues) were collected aseptically and kept at -20°C till further use.

Isolation of *Listeria spp.*

Isolation of *Listeria* species was based on two fold enrichment procedure as recommended by USDA 2009 and as described by Loncarevic, *et al.*, (2006) with suitable modifications. Briefly, approximately 10 g of samples were directly inoculated into 90 ml of University of Vermont-1 (UVM-1, Himedia Labs, Mumbai, India) and incubated overnight at 30°C. The enriched UVM-1 inoculum (0.1 ml) was then transferred to 10 ml of Fraser broth and incubated overnight at 30°C. The inoculum from the enriched Fraser

broth was streaked on oxford agar and inoculated plates were incubated at 37 °C for 48 h. The typical small, round greyish blackish colonies of about 0.5 mm diameter surrounded by diffuse black zone of aesculin hydrolysis were presumed to be *Listeria* spp (Fig.1). The presumed colonies of *Listeria* (at least 3/plate) were further confirmed.

Confirmation of the isolates

These isolates were further subjected to different biochemical tests according to standard procedure described by Cruickshank *et al.*, (1975) and Cowan (1977). Typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20–25 °C, methyl red–Voges Proskauer (MR–VP) reactions, CAMP test with *Staphylococcus aureus* and *Rodococcus equi*, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and α -methyl-D-mannoside) and hemolysis on 5% sheep blood agar (SBA). The isolates were further tested for their pathogenicity.

Pathogenicity test

Phosphatidylinositol-specific phospholipase C (PI-PLC) assay. All the biochemically characterized *Listeria* isolates were assayed for PI-PLC activity as per the method of Notermans *et al.*, (1991) and Ottoviani *et al.*, (1997) with certain modifications.

The *Listeria* isolates were grown overnight onto L. mono Confirmatory Agar Base supplemented with L. mono Selective Supplement I & II and L. mono Enrichment Supplement II at 35-37°C. Phospholipase C enzyme produced by *L. monocytogenes* and *L. ivanovii* hydrolyses the purified substrate (FD227) added to the medium and results in the formation of an opaque halo around the colonies (Fig.2 &3).

It also contains alpha-Methyl D-mannoside, its fermentation is shown by *L. monocytogenes*. Thus, Differentiation between *L. monocytogenes* and *L. ivanovii* is achieved on the basis of alpha -Methyl D-mannoside utilization and PIPLC activity.

Determination of total nitrate

The total nitrate and nitrite estimation in hypothalamus, follicular fluid, ovarian tissue, fallopian tube and uterus was carried out as per the modified method described by Sastry *et al.*, (2002). The method performed is as follows: Reproductive tissues (ovarian, oviductal and uterine tissues) and hypothalamus were weighed and homogenized in ice-cold 1.0M PBS with a pH of 7.4. The homogenate was then subjected to centrifugation at 4000 g for 10 minutes at 4°C. The supernatant was then collected in fresh tubes. Alongside test samples, standards were also processed. 100µL of supernatant (test sample) and standard solutions were taken in test tubes and to them 400 µL of 50 mM carbonate buffer was added to each test tube.

Activation of copper-cadmium alloy

Copper-cadmium alloy filings were washed twice with 100 ml of deionised distilled water in a 150 ml Erlenmeyer flask. After discarding the supernatant, the filings were washed with 100 ml 0.5N HCl. This washing removed the hydroxide of cadmium Cd(OH)₂, which resulted in a change in texture and color. The filings appeared gray and slightly buoyant. These activated filings are quickly washed twice with 100 ml of 0.1N HCl and stored in 50 ml of 0.1M HCl at 2-8°C till further use. After activation approximately 150 mg of dried copper-cadmium alloy was added in the test and standard sample tubes followed by incubation of test tube for 1hr at 37°C with thorough shaking. This incubation

causes full conversion of nitrate to nitrite. After incubation, 100µL of 0.35 M Sodium hydroxide was added to halt the reaction; followed by adding 400 µL of 120 mM Zinc sulphate solution on a vortex mixture. Zinc sulphate leads to deproteinization (white precipitation was seen). The test tubes were allowed to stand for 10 minutes. The tubes were centrifuged at 4000g for 10minutes at room temperature. Precipitate settled down and clear transparent supernatant stood up. Then these samples were subjected to macro assay.

Macro-assay

Aliquots (500µL) of clear supernatant were transferred to another test tube and 250µL of 1.0% Sulfanilamide (prepared in 3N HCl) and 250µL of 0.1% N-naphthylethylenediamine (prepared in water) were added with shaking. After 10minutes the absorbance was measured at 545 nm against a blank containing the same concentration of ingredients without any test sample.

Results and Discussion

Listeria spp. are ubiquitous organisms and an increasing number of recent reports show prevalence of *L. monocytogenes*. *Listeria* spp. have been isolated from various different environments, including soil, surface water, vegetation, sewage, animal feeds, farm environments, and food-processing environments (Lotfollahi *et al.*, 2017). Of the six bacteria species within the genus, *L. ivanovii* is one of the two pathogenic species (the other being *L. monocytogenes*). It behaves like *L. monocytogenes*, but is found almost exclusively in ruminants (mainly sheep).

In the present study, out of (n=78) serum and organs (comprising of 26 uterus, 26 ovaries and 26 brain) samples, *L. monocytogenes* was detected in 5 samples i.e. 3 in uterus, 1 in ovary and 1 in brain, with the over-all prevalence of 6.41%. On other hand, *L. ivanovii* was isolated only in 3 uterus sample with the prevalence of 3.85%.

Table.1 Concentration of Nitric Oxide (NO) and presence and absence of *Listeria* spp. in corresponding tissues

Sample number	Uterus		Ovary		Brain		NO level in Serum
	NO level	Isolation	NO level	Isolation	NO level	Isolation	
Mean of 17 negative samples	17.98	-	16.35	-	15.74	-	26.82
7	51.60	<i>L. mono</i>	40.80	<i>L. mono</i>	40.00	<i>L. mono</i>	44.80
8	16.00	-	30.00	<i>L. spp.</i>	35.60	-	40.80
10	45.60	<i>L. ivano</i>	31.60	-	38.80	-	55.60
17	38.40	<i>L. ivano</i>	22.40	-	20.40	-	44.80
18	36.80	<i>L. mono</i>	15.20	-	32.40	-	146.80
20	18.00	-	20.00	-	35.20	<i>L. spp.</i>	16.40
22	46.40	<i>L. ivano</i>	11.60	-	13.20	-	74.00
23	46.80	<i>L. spp.</i>	13.60	-	10.80	-	39.60
27	50.40	<i>L. mono</i>	23.60	-	8.00	-	46.00

Fig.1 *Listeria spp.* on oxford agar



Fig.2 *Listeria monocytogenes* on L. mono confirmatory agar



Fig.3 *Listeria ivanovii* on L. mono confirmatory agar



The prevalence of other *Listeria spp.* in the sample was 3.85%. All the isolates were gram positive, cocco-bacillus, positive for catalase, Methyl Red and Voges-Proskauer reaction. They showed negative reaction for indole, oxidase, H₂S, urea hydrolysis, nitrate reduction and gelatin liquefaction. But showed characteristic tumbling motility when kept in the broth at 25°C for 18-24 hrs. In our study 8 samples showed typical β -haemolysis when streaked on HBA (5%), for 24-48 hr at 37°C. But no hemolysis pattern was observed in 3 isolates. All the 8 positive haemolytic isolates were subjected to CAMP test and

Phosphatidylinositol-specific phospholipase C (PIPLC) assay. 5 isolates showed enhancement on *Staphylococcus aureus* streak and 3 on *R. equi* streak on HBA plate. Similar results were obtained on PIPLC assay, thus 5 isolated showing yellow colonies and halo zone in PIPLC assay were confirmed as *L. monocytogenes* and the 3 isolates showing purple colonies with halo were confirmed as *L. ivanovii*.

The prevalence recorded in present investigation is lower as compared to the reported 30% prevalence of *Listeria spp.* from

69 brain samples of goats collected at the time of post-mortem (Shivasharanappa *et al.*, 2014). *L. monocytogenes* can also be isolated from the surface and underground waters, improperly fermented silage, sewage sludge, slaughter wastes, animal and human faeces, foodstuffs, and food industry plants (Ivanek *et al.*, 2006). In the current study it was found that there was high concentration or expression of NO in *Listeria* infected tissues in comparison to the tissues where *Listeria* was not isolated (Table.1).

Our findings are similar to the reports given by Brosnan *et al.*, (1994) and Moncada *et al.*, (1991), which suggests the involvement of NO (central nervous system) in pathological sequelae. It was found that (sample 7), there is increase in nitric oxide concentration in the brains of *Listeria* infected sheep. Therefore, nitric oxide produced by neutrophils and macrophages may play an important role in eliminating *L. monocytogenes* and in the histopathology of brain tissue. Even though *Listeria* spread to other organs but powerful anti-listerial mechanisms of NO led to a complete elimination of *L. monocytogenes* from the brain and other organs. There was isolation of *L. monocytogenes* from sample 18 (uterus), but the concentration of NO was found very high in the serum in comparison to the tissue. It may suggest chronic infection in the uterus, but the high NO level of serum does not result in spread of infection to other organs. However some earlier workers suggest that the role of NO as anti-listerial activity is controversial (Ohya *et al.*, 1998; Samsom *et al.*, (1996). But Remer *et al.*, (2001) had reported that there is neutralize of *L. monocytogenes* by the production of NO from macrophages. The role of NO generation in ruminant listeriosis remains to be studied. Another question raises that whether NO generation is essential for the elimination of *L. monocytogenes* in the brain. However some studies show that killing of *L. monocytogenes*

in-vitro was dependant on NO (Fehr *et al.*, 1997). Although *L. monocytogenes* are by far the leading cause of human and animal's listeriosis, and its role of high NO in infected sheep. But, our report shows that *L. ivanovii* infected can also cause enhance expression of NO levels. As we know, *Listeriosis* causes sudden death in animals, thus the functional role of nitric oxide (either iNOS or nNOS) in *Listeria* spp. affected animals needs to be further studied.

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