

**Studies on the Prevalence of *Listeria* spp. in  
Sheep of Kashmir Valley**

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(2007-V-73-M)



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**Studies on the Prevalence of *Listeria* spp. in  
Sheep of Kashmir Valley**

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(2007-V-73-M)



***THESIS***

*Submitted to*

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(Veterinary Public Health)**

**2009**



**Sher-e-Kashmir**  
**University of Agricultural Sciences & Technology of Kashmir**  
**Division of Veterinary Public Health,**  
**Shuhama Campus Srinagar– 190 006**  
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**Certificate – I**

This is to certify that the thesis entitled, “**Studies on the Prevalence of *Listeria* spp. in Sheep of Kashmir Valley**” submitted in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Sciences (Veterinary Public Health)**, to the **Faculty of Postgraduate Studies, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Dr. Jaffar Sharif (Regd. No. 2007-V-73-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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### ABSTRACT

Prevalence of listeric abortions in organized and unorganized sheep farms of Kashmir valley was studied during 2008-2009. A total 52 cases of abortions/stillbirths were screened for isolation of *Listeria* species. *Listeria monocytogenes* was the only species of Genus *Listeria* isolated from cases of abortions/stillbirths under study. An overall prevalence of 15.38 per cent of *listeriosis* was recorded in these farms. The prevalence was higher in organized (18.51%) than in the un-organized farms (12.00%). Out of a total 8 isolates recovered, 4 were isolated from only brain tissues, 2 from both brain and livers and one each from liver and stomach contents. All the isolates produced marked degree of beta haemolysis on 10 per cent sheep

blood agar plates in 24-36 hr. The 18 hr old broth culture produced kerato-conjunctivitis and monocytosis in healthy rabbits within 2- 6 days on intra-ocular instillation. Inoculation of viable *Listeria monocytogenes* into two pregnant does, on 20<sup>th</sup> day of gestation by intra-peritoneal route, resulted in abortion within 48 hr post inoculation. All the isolates of *Listeria monocytogenes* under study, were sensitive to gentamicin, doxycycline, ampicillin, tetracycline and pencillin G in order and resistant to nalidixic acid, co-trimaxozole, ciprofloxacin, chloramphenicol, erythromycin and lemloxacin.

**Key words:** *Listeria monocytogenes*, Prevalence, Abortion, Still births

Signature of student

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**Jaffar Sharif**

**Place :**

**Dated:**

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## CHAPTER – 1

### INTRODUCTION

*Listeriosis* is an acute infectious disease of animals and humans caused by bacteria belonging to the genus *Listeria*, which presently includes seven species. Among these *Listeria monocytogenes* and *Listeria ivanovii* are regarded as pathogenic species whereas *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria murrayi* and *Listeria grayi* are non pathogenic species (Seeliger and Jones, 1986; Mcluachlin, 1987). Disease occurs mainly in temperate climates and is much less common in tropical and sub tropical areas. Global occurrence of *listeriosis* has been reported

from almost all species of domestic animals as well as from many species of poultry, fish, wild animals and rodents. The disease is of great economic importance in sheep, goats and cattle and less so in pigs and poultry. Rodents, particularly rabbits and guinea pigs, are highly susceptible to natural infection. The role of *Listeria monocytogenes* in causing meningo-encephalitis, septicemia, endocarditis, abortion, cervicitis, diarrhoea, mastitis, keratoconjunctivitis has been established beyond doubt. (Gitter *et al.*, 1986). *Listeriosis* is a significant public health problem (Roucort and Berche, 1987). Milk, dairy products, meat, poultry, vegetables, salad and sea foods have been found to be sources of contamination (WHO, 1988). In humans, it leads to septicemia, abortion, stillbirths, meningitis and meningo-encephalitis especially in immuno-compromised individuals and those in contact with animals (Lessing *et al.*, 1994). *Listeria monocytogenes*, widely recognized as a cause of abortions in domestic animals, was first described by Murray *et al.* (1926) who named it as *Bacterium monocytogenes* because of the characteristic monocytosis exhibited in the infected rabbits and guinea pigs. *Listeriosis* in animals is diagnosed on the basis of clinical symptoms, isolation of the organisms from clinical specimen, animal inoculation tests or inoculation in 10 days old chicken embryos through chorioallantoic route, and or by serodiagnostic tests such as serum agglutination, CFT, HA, ELISA. The

organism is invariably sensitive to a wide range of antibiotics like ampicillin, amoxycillin, tetracycline, chloromphenicol, *B. lactam* antibiotics etc. Disease in man can be prevented by controlling *listeriosis* in animals through culling of infected animals, care during preparation and usage of silage and in endemic areas by vaccination coupled with proper handling of cases of abortions in animals and avoiding consumption of contaminated foods.

Kashmir with its vast high land pastures and land to population ratio is an excellent place for rearing and management of farm animals especially sheep under migratory extensive production system. The highest setback in the development of sheep industry in this part is the management of disease conditions especially those that cause abortions and stillbirths in these animals. *Listeriosis* is one such disease which is responsible for heavy lamb mortality besides causing abortions and stillbirths in ewes and resultant heavy economic losses. Keeping in view the zoonotic and economic importance of the disease the studies were taken with the following objectives:

- (1) To study the prevalence of *Listeria* spp. in sheep of Kashmir valley.
- (2) To isolate *Listeria* species from cases of abortions and stillbirths

by microbiological techniques.

- (3) To identify the *Listeria* species by Polymerase Chain Reaction.
- (4) To study the antibiotic sensitivity pattern of the *Listeria* isolates.  
recovered from the clinical cases.

## CHAPTER – 2

### REVIEW OF LITERATURE

*Listeriosis* is an important bacterial zoonosis caused by *Listeria monocytogenes* and *Listeria ivanovii*, the only recognized pathogenic species of the Genus *Listeria*. The disease is prevalent through out the world with a wide host range including mammals, birds and fishes (Sonnenwirth, 1980). The disease been reported in all domesticated species including bovines (Fedio *et al.*, 1990); capro-ovines (Reiter *et al.*, 1989); porcines (Lopez *et al.*, 1989), and poultry (Ojeniyi *et al.*, 1996). *Listeria monocytogenes* is the major pathogenic species affecting both animals and

man (Roucort and Seeliger, 1985). Animal *listeriosis* is manifested by septicemia, mastitis, encephalitis, meningitis, endometritis, stillbirths and abortions. Among domesticated animals, sheep is the most commonly affected species (Anonymous, 1992).

History of *listeriosis* dates back to the isolation of the organism from liver of rabbit by Hulphers (1911) who named it as *Bacillus hepatis*. Fifteen years later Murray *et al.* (1926) isolated a bacteria similar to one isolated by Hulphers from rabbits and pigs causing monocytosis. Pirie (1927) isolated a bacillus from liver of gerbils and named it as *Listerella hepatolytica* in honour of surgeon Joseph Lister and thereafter *Listerella* was included in the 6<sup>th</sup> edition of Bergey's Manual of Determinative Bacteriology. The organism was however, renamed as *Listeria* in 1940 for taxonomic reasons (Gray and Killinger, 1966). *Listeria monocytogenes* was recovered for the first time from human beings in Denmark by Nyfeldt (Gray and Killinger, 1966). However, it was the recognition in the 1980's of *listeriosis* as a food-borne human disease and *Listeria monocytogenes* as an important emerging food borne pathogen by World Health Organization (WHO) in the year 1988 that resulted in intense research activity (Low and Donachie, 1997). *Listeria ivanovii* (formerly known as *Listeria monocytogenes* serotype 5) was first isolated in Bulgaria in 1955 from lambs with congenital *listeriosis*

(Ivanov, 1962).

## **2.1 Prevalence in ovines**

Hindmarsh and Blumer (1932) were first to report listeric abortions in sheep from Australia. Mathiesen and Wray (1969) described an outbreak of *listeriosis* in ewes from Scotland. Broadbent (1972) recorded an abortion rate of 20 per cent due to *Listeria monocytogenes* in 23 ovine flocks in Australia. The macroscopic lesions were found in the aborted fetuses or dead neonates from which *Listeria monocytogenes* was isolated. Dennis (1966) confirmed *Listeria monocytogenes* as the cause of death of 40 lambs from 15 outbreaks of abortion. Among these, 50 per cent of the lambs succumbed in-utero, 10 per cent during parturition and 40 per cent post-partum.

*Listeria monocytogenes* associated abortion outbreak in ewes was reported by Macleod *et al.* (1974) from Britain. An abortion rate of 15 per cent was recorded in two ovine flocks. The dead fetuses were oedematous with excessive fluid in the body cavities.

Antic *et al.* (1989) studied the frequency of abortions in five farms over two years (1987-89) and observed abortion rates of 3.2 per cent in 1987 and 2.9 per cent in 1988. *Listeria monocytogenes* was isolated from all the aborted fetuses. In France 35 per cent seroprevalence of *listeriosis* in

sheep has been reported (Pasa *et al.*, 2003).

Out of 428 Listeric outbreaks during the course of two years in France, 108 were recorded in sheep with major clinical manifestations of abortion (Vaissaire, 2000). Pasa *et al.* (2003) recorded a seroprevalence of 35 per cent due to *Listeria monocytogenes*. Nightingale *et al.* (2004) recorded a prevalence of 32.9 per cent in sheep while studying the ecology and transmission of *Listeria monocytogenes* infecting ruminants and the farm environment.

Schwab *et al.* (2004) reported the disease in 5 animals (2 cows, 2 sheep and 1 goat) from Brazil.

The first authentic case of Listeric abortion in any species of domesticated animal from India was reported by Dhanda *et al.* (1959) in a ewe. In 1977-78, 111 abortions out of 800 lambings in ovines of Jammu and Kashmir were reported by Vishwanathan and Uppal (1981). The abortions occurred mostly towards the end of gestation and were not accompanied by any characteristic signs in the aborting ewes. *Listeria monocytogenes* 4b was isolated from the stomach contents of one of 22 aborted fetuses. *Listeria monocytogenes* accounted for 20 per cent of the abortions.

The seroprevalence for *listeriosis* in infertile ewes in Himachal

Pradesh has been found to be 18.3 per cent (Nigam *et al.*, 1996). The seroprevalence of *listeriosis* has been found to be 83.3 per cent in western India (Butchaiah and Khera 1981), 18.75 per cent in sheep in North Gujrat (Vela *et al.*, 2005).

In Himachal Pradesh studies on migratory flocks of 216 sheep revealed the prevalence of listeric abortions to be 9.1 per cent (Nigam *et al.*, 1996).

Chand and Sadana (1999) reported an outbreak of abortion (23%) due to *Listeria* spp. in a flock of 254 cross bred sheep maintained at Hissar Agricultural University.

Willayat *et al.* (2005) reported abortions due to *Listeria monocytogenes* in sheep in Kashmir. *Listeria monocytogenes* has also been isolated from semen of rams in Kashmir (Islam *et al.*, 2005).

## **2.2 Prevalence in human beings**

Although an uncommon cause of illness in the human population, *listeriosis* in man is often severe with mortality rates reaching up to 50 per cent. The incidence of *listeriosis* in general population is 0.7 per 100,000; while in the pregnant women is 12 per 100,000 i.e. a 17-fold increase (Southwick and Purich, 1996). The overall seropositivity for human *listeriosis* has been reported to vary from 1.6 per cent in Austria (Sixl *et al.*,

1978); 6.76 per cent in province of Guano, Italy (Caramello *et al.*, 1975) and 21 to 37 per cent in Germany (Nass and Ortel, 1977). The disease is asymptomatic in the pregnant women and occurs as a mild influenza like syndrome (Tridente *et al.*, 1998). Urinary tract infections are also noticed in some patients (Benshushan *et al.*, 2002). Following delivery, mothers of infected babies may shed *Listeria monocytogenes* for 7-10 days in their vaginal secretions or urine (MDPH, 2001). Latent *listeriosis* in pregnant women leads to habitual abortions, intrauterine deaths, and fetal malformations (Winkhaus-Schindl, 1966; Romana, 1989).

Reports of *listeriosis* from humans in India are scanty, either because of failure to identify the pathogen, its rarity, improper isolation techniques, low incidence rate or lack of awareness. Krishna *et al.* (1996) isolated *Listeria monocytogenes* from the cervix of 14 per cent of 150 patients with a past history of abortion, miscarriage, stillbirth, or neonatal deaths. Two cases of human *listeriosis* have been recorded in India by Roucort (1991). Clinical manifestation of the disease is mostly meningitis or more rarely as encephalitis (Low and Donachie, 1997). However, generalized infection can result in spontaneous abortion, stillbirths or infection in the infants (Seeliger and Finger, 1983).

### **2.3 *Listeriosis***

*Listeriosis* is a serious invasive bacterial zoonosis caused by species of the genus *Listeria*. The genus *Listeria* comprises of six species namely *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi* among which *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri* are hemolytic. *Listeria monocytogenes* is a gram-positive, nonsporulating, facultative anaerobe measuring 1-2  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  in width with bacteria often lying parallel to one another in palisades, and thread like forms. Growth occurs between 3 and 50°C but the optimum temperature is 30-37°C. Typical tumbling motility is seen in 18 hour old broth cultures at 20-25°C (Topley and Wilson, 1990). Growth is absent or scanty in complete anaerobic condition and the multiplication is inhibited at pH values lower than 5.6. The bacterial colonies are smooth, slightly flattened and milky white by reflected light (Gray and Killinger, 1966). The organism is catalase positive, oxidase negative and produces a zone of hemolysis on blood agar (Farber and Peterkin, 1991).

Based on the somatic (O) and flageller (H) antigens, 12 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7) of *Listeria monocytogenes* and one (5) of *Listeria ivanovii* have been recognized (Seeliger and Jones, 1986; Kathariou, 2002). *Listeria monocytogenes* is

widely distributed in the environment, and has been isolated from numerous sites including soil, sewage water and decaying plant material (especially poorly fermented silage). The viability of the agent is remarkable as it can survive in soil or silage for more than two years. It has been found in the gut of more than five per cent of the normal healthy people (OIE, 2005). For this reason, exposure to this bacterium is considered as unavoidable (Association of Medical Microbiologists, 1995).

*Listeria monocytogenes* is the major pathogenic species affecting both animals and human beings (Roucort and Seeliger, 1985., Mc lauchlin, 1987). The disease is also known as caprine bacterial encephalitis (in goats), circling disease and listerial abortion in animals. It commonly affects the pregnant uterus, the central nervous system and the circulatory system resulting in abortion, stillbirth, meningitis, meningoencephalitis and septicaemia, especially in immunocompromised individuals and those in contact with animals. Of late, the disease has assumed great importance as an emerging food-borne zoonosis with a common source epidemic potential, and a nagging public health hazard. There are more than 350 zoonotic diseases known today, but *listeriosis* is given special attention due to the unique and changing concept of zoonoses. In the early 1980s, *listeriosis* was classified under anthroozoonoses, which was changed to

amphixenoses in the late 1990s. It lacks its true definition of zoonosis because of involvement of inanimate reservoir food as the major cause of *listeriosis*. Feeding has been incriminated as the predisposing factor in winter for *listeriosis* in animals especially sheep. Majority of the cases have been reported in winter following consumption of silage contaminated with *Listeria monocytogenes*. *Listeriosis* is somewhat seasonal, most cases having been reported in winter. Similarly, cases of Listeric abortions are high during February and March owing to seasonal lambing. No breed differences have been noticed as far as the occurrence of the infection is concerned. However it has been reported mainly from young or pregnant animals and also from animals with reproductive disorders (Butchaiah and Khera, 1982; Srivastava *et al.*, 1985). The high occurrence of the disease in young animals may be related to their underdeveloped immune systems, whereas the fundamental physiological and immunological changes that occur in pregnant animals may be contributing to the susceptibility of such animals (Low and Donachie, 1997).

Listeric infections and abortions usually develop in the late winter or early spring. Abortions are commonly recognized in the last trimester of pregnancy, frequently in the absence of other clinical signs (Kapur and Sadana, 1974; Jubb *et al.*, 1985). *Listeric* abortions caused by *Listeria*

*monocytogenes* occur in ruminants especially sheep (Gray and Killinger, 1966; Njoku *et al.*, 1972). *Listeria ivanovii* has also been frequently seen to be associated with abortion in sheep (Ivanov, 1962; Chand and Sadana, 1999) and extremely rare as a cause of other conditions like encephalitis, endocarditis, mastitis *etc.* In sheep abortion could occur from 12<sup>th</sup> week of pregnancy with a majority of losses in the last four weeks of gestation and the birth of full term dead lambs. The after death is usually retained and there is blood tinged vaginal discharge for several days (Diplock, 1957; Mc Donald, 1967; Gitter *et al.*, 1986). Affected ewes generally do not show any premonitory signs (Broadbent, 1972). Metritis, retained placenta and fatal septicemia have been described as a sequel of *Listeric* abortions ( Paulsen and Moule, 1953; Diplock, 1957) The aborting ewes abandon lambs soon after delivery and *Listeric* infection has not been found to cause significant mortality among lambs that survive more than 24 hours after birth (Broadbent, 1972). The stage of gestation at which a ewe is infected is significant in determining the subsequent course of events (Smith *et al.*, 1968).

The septicemic form of *listeriosis* is uncommon and generally occurs in the neonate as an extension of intra-uterine infection. The most consistent lesion is focal necrosis with pin point greyish white nodules

throughout the liver. Occasional massive outbreaks of septicemia involving pregnant ewes have been described (Low and Renton, 1985). In adult sheep, marked enteritis with extensive haemorrhage affecting the abomasal folds, ulceration of the abomasal and intestinal mucosae and abscessation of Peyer's patches as a result of infection of *Listeria monocytogenes* has been recognized (Low *et al.*, 1997).

Encephalitic *listeriosis* is more prevalent among ruminants, especially sheep, with an attack rate of approximately 10-12 per cent and mortality rate of 5-10 per cent (Gitter *et al.*, 1986)

The first description of Listeric encephalitis was a widespread neurological disease of sheep in New Zealand locally known as 'circling disease' (Gill, 1931). The encephalitic form results from trigeminal nerve infection consequent to abrasions of the buccal mucosa with feed or infection of teeth cavities.

#### **2.4 Foodborne *Listeriosis***

*Listeriosis* is a significant public health problem (Roucort and Berche, 1987). Milk, dairy products, meat, poultry, vegetables, salad and sea foods have been found to be sources of contamination (W.H.O., 1988). The first confirmed role of food in the transmission of Listeric infection was provided by the outbreak that occurred in 1977 in Boston (USA) in

which 20 persons were reported to have developed *listeriosis* by eating contaminated raw celery of tomatoes and lettuce (Ho *et al.*, 1986). The Mexican style soft cheese contaminated with *Listeria monocytogenes* serotype 4b was involved in a large outbreak in Los Angeles, California USA in 1985 (James *et al.*, 1985). In another outbreak in Switzerland in 1987 soft cheese was traced to be responsible from which *Listeria monocytogenes* serotype 4b was isolated (Piffareti *et al.*, 1989). Infection can also be transmitted by contact with infected animals, usually resulting in cutaneous lesions (McLauchlin and Low, 1994) or by cross-infection during the neonatal period (Schlech, 1991). Primary cutaneous *listeriosis* has been reported in veterinarians (Regan *et al.*, 2005) as a result of direct transmission.

Ho *et al.* (1986) reported that raw vegetable was responsible for an outbreak of *listeriosis* in September and October 1979 in hospitalized patients and 15 out of 20 patients were known to have acquired infection due to ingestion of tuna fish, chicken salad and cheese. Barbuddhe *et al.* (2000) have isolated *Listeria monocytogenes* from raw milk of goat, sheep, buffalo; Bhilegaonkar *et al.*, 1997(raw cow milk). Taludhar and Sharma (1997) have isolated *Listeria monocytogenes* from cheese. Brahmabhatt and Anjaria (1993) have isolated *Listeria* spp. from raw sheep meat and goat

meat. *Listeria monocytogenes* was isolated from raw milk supplies collected at Ranchi, Jharkhand (Yadava *et al.*, 1985). *Listeria monocytogenes* was also recovered from 8.1 per cent of samples of raw milk in Bareilly, Uttar Pradesh (Bhilegankar *et al.*, 1997).

## **2.5 Laboratory diagnosis**

*Listeriae* are presumptively identified on the basis of their morphology and characteristic tumbling motility at 22°C besides production of catalase and oxidase and by demonstration of haemolysis in RBCs of sheep in blood agar (SBA), (Farber and Peterkin, 1991). But the confirmatory diagnosis is made only by isolation and identification of the pathogen (Rocourt *et al.*, 1983). The organism is isolated from clinical specimens, such as blood, cerebrospinal fluid (CSF), vaginal secretions, the meconium of the newborn or foetus in cases of abortion, faeces, vomitus and also from food stuffs and animal feeds (Gupta *et al.*, 2003). The recovery of *Listeria monocytogenes* and *Listeria ivanovii* from food and environmental samples requires the use of enrichment cultures followed by selective plating (Curtis and Lee, 1995).

To isolate the organism, two methods are employed in the United States, the Food and Drug Administration (FDA) method and the United States Department of Agriculture (USDA) method (Lovett *et al.*, 1987;

Lovett and Hitchens, 1988; Mc Clain and Lee, 1988).

Different enrichment and selective media for detection of *Listeria* spp. have been reviewed by Curtis and Lee 1995. For many years, the only method available was the cold enrichment technique advocated by Gray *et al.*, 1948. Later, increased interest in *Listeria monocytogenes* as a food-borne pathogen led to the development of many selective broths like L-PALCAMY (Polymixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk) (Van Netten *et al.*, 1989), University of Vermont-I (UVM I) (Donnelly and Baigent, 1986), University of Vermont-II (UVM II) (McClain and Lee, 1988) and Fraser broth (Fraser and Sperber, 1988).

The use of complete UVM, a two step procedure employing UVM-1 and UVM-2 is found to be more effective than that of Holman medium or UVM-1 alone (Ralovich, 1989). UVM and Listeria enrichment broth (LEB) broth allow better recovery of heat-injured cells of *Listeria monocytogenes* (Bailey *et al.*, 1990).

ALOA (Agar Listeria according to Ottaviani and Agosti) is a chromogenic medium, which is not only selective for the isolation of *Listeria* species but also allows direct differentiation of pathogenic *Listeria monocytogenes* and *Listeria ivanovii* in presence of other *Listeriae* and various background flora. The ALOA medium comprises of a chromogenic

compound 5-bromo-4-chloro-3-indoxyl- $\beta$ -d-glucopyranoside, acting as a substrate for the detection of  $\beta$ -d glucosidase present in all *Listeria* species. The differential detection of *Listeria monocytogenes* and *Listeria ivanovii* is based on the production of PI-PLC by the pathogen which hydrolyzes the substrate L- $\alpha$ -phosphatidylinositol and produces opaque clear-cut halo surrounding the colonies (Ottaviani *et al.*, 1997; Reissbrodt *et al.*, 2004).

A new test DIM (differentiation of *innocua* and *monocytogenes*) based on detection of acrylamidase which is present in all *Listeria innocua* strains and also in majority of other non-*Listeria monocytogenes* listerial strains but absent in *Listeria monocytogenes* has been devised. (Bille *et al.*, 1992). However, despite the availability of alternative identification techniques, conventional and hemolytic reactions are still the most common techniques used for identification of *Listeriae* (Mclauchlin 1987; Chen and Chang, 1996).

*Listeria monocytogenes* is heterogeneous in respect of its virulence (Hof and Rocourt, 1991), which is thought to be a multifactorial phenomenon (Ralovich, 1984). Differentiation between hemolytic and pathogenic species (*Listeria monocytogenes* and *Listeria ivanovii*) and non-hemolytic and non-pathogenic species (*Listeria innocua* and *Listeria welshimeri*) becomes possible by the hemolytic activity of the former.

Positive haemolysis demonstration of virulent *Listeria* can be done either by simple agar slab technique or the Christie Atkins Munch Petersen (CAMP) test. A positive CAMP reaction, fermentation of rhamnose and non-fermentation of xylose are the three in vitro tests used to identify pathogenic *Listeria* spp. However, there is one species, *i.e.*, *Listeria seeligeri*, which is haemolytic but non-pathogenic (Seeliger, 1981).

Phosphatidylinositol-specific phospholipase C (PI-PLC)-based assay has been reported to be more reliable to discriminate pathogenic and haemolytic *Listeria* spp., *i.e.*, *Listeria monocytogenes* and *Listeria ivanovii* from nonpathogenic but haemolytic spp., *i.e.*, *Listeria seeligeri* (Notermans *et al.*, 1991b). However, in order to judge the potential of an in vitro pathogenicity test it needs to be compared with in vivo pathogenicity tests.

Virulence of *Listeria monocytogenes* for man has been correlated with pathogenicity in mice (Mainou-Fowler *et al.*, 1988), particularly in immuno-compromised mice (Stelma *et al.*, 1987). However, death in normal mice has been found to give clear differentiation between virulent and non-virulent strain following single inoculum (Tabouret *et al.*, 1991). All the virulent strains tested showed 100 per cent mortality by aerosol infection (Chen and Chang, 1996), while virulent and avirulent strains were indistinguishable by gastric intubation (Bracegirdle *et al.*, 1994).

On inoculation of *Listeria* species in chick embryo through chorioallantoic (CAM) route, pathogenic strains cause death of embryo within 72 hours while nonpathogenic fail to do so (Terplan and Steinmeyer, 1989) and the test has been reported to agree with mouse bio-assay (Notermans *et al.*, 1991a). Yolk sac route inoculation has been found to be less suitable than the CAM challenge for assessing virulence because of non-specific deaths encountered in the former route (Notermans *et al.*, 1991a).

Serological tests lack sensitivity and specificity in a number of formats, including ELISA, complement fixation and micro agglutination tests. Considerable cross-reactivity with antigenic determinants of various Gram-positive organisms is one reason. Besides regular exposure of animals and humans to *Listeria monocytogenes* is very common as it is a ubiquitous organism.

It is important to have a reliable serological test based on *Listeria* specific antigen that may solve the problem of cross-reactivity of Listerial antigens. So it has been suggested that the diagnostic method(s) for rapid and reliable diagnosis of pathogenic *Listeria* species should ideally be based on virulence marker(s) (Notermans *et al.*, 1991b). Of late, the potential of *Listeriolysin-O* as reliable virulence marker has been assessed,

as LLO is a target antigen of listerial immunity (Archie Bouwer *et al.*, 1992, Harty and Bevan, 1992). However, LLO is antigenically related to a number of cytolysins, including streptolysin O (SLO) from *Streptococcus pyogenes*, pneumolysin from *S. pneumoniae* and perfringolysin from *Clostridium perfringens*. Problems of cross-reactivity of anti-LLO antibodies with these cytolysins, particularly SLO and pneumolysin, have hampered the development of specific reliable serological tests based on the detection of anti-LLO antibodies. In addition, anti-LLO antibodies have been found in a proportion of healthy individuals and patients with other bacterial, fungal or viral infections (27%, all combined), although at lower titres than in patients with *listeriosis*. (Tizard, 1982).

Polymerase chain reaction (PCR) has been used increasingly as an additional tool for the identification of microorganisms because of its rapid, sensitive and specific detection. This facilitates exponential amplification of a pre-selected region of DNA (Mullis and Faloona, 1987) and thus represents a highly specific and sensitive detection technique. Norton (2002) has reviewed nucleic acid amplification-based methodology, specifically polymerase chain reaction (PCR)-based assays, for the detection of *Listeria monocytogenes* in food and environmental samples.

Several virulence-associated genes have been sequenced and used as

PCR-targets for the detection of *Listeria monocytogenes*, for example, the *Listeriolysin O (hyl A)* gene (Bessensen *et al.*, 1990; Border *et al.*, 1990; Dencer and Boychuk, 1991; Rossen *et al.*, 1991), the Dth 18 gene (Wernars *et al.*, 1991), the *iapA* gene (Jaton *et al.*, 1992; Kohler *et al.*, 1990) and the *plc A* gene (Notermans *et al.*, 1991b). *Listeriolysin O* (LLO) encoded by *hyl A* gene has been regarded as the most important virulence factor

Recently, multiplex PCR targeting virulence genes of *Listeria monocytogenes* (*plc A*, *prf A* and *hyl A* genes) and *Listeria ivanovii* (*plc A*, *prf A* and *act A* genes) has been employed (Rawool, 2004).

Among newer diagnostic methods for detection of *Listeria monocytogenes*, fluorescent antibody technique (Watson and Eveland; 1965) and peroxide anti-peroxide staining method exist (Domingo *et al.*, 1986) but cross-reaction with other bacteria was observed due to the use of polyclonal sera. Specific monoclonal antibodies may be useful (McLauchlin *et al.*, 1988) but these have not been used in veterinary studies (Low and Donachie, 1997).

Other rapid methods include flow cytometry (Donnelly and Baigent, 1986), electrical impedance (Hancock *et al.*, 1993) and immunomagnetic separation (Skjerye *et al.*, 1990). In a recent novel approach, an ELISA mediated PCR technique was developed to detect and quantify *Listeria*

*monocytogenes* in food products (Wang *et al.*, 1999). ). In a recent novel approach, an ELISA mediated PCR technique was developed to detect and quantify *Listeria monocytogenes* in food products (Wang *et al.*, 1999).

## 2.6 Antibiotic Sensitivity

All the strains of *Listeria* spp. are reportedly sensitive to ampicillin, and this, in combination with an aminoglycoside, remains the treatment of choice *in vivo* (MacGowan, 1990). Apart from the reported plasmid-encoded resistance to chloramphenicol, erythromycin, streptomycin and tetracycline (Poyart-Salmeron and Trieu-court, 1990; Quentin *et al.*, 1990; Hadorn and Hachler, 1993), strains are universally sensitive to amikacin, amoxicillin, ampicillin, azlocillin, ciprofloxacin, chloramphenicol, clindamycin, coumermycin, doxycyclin, enoxacin, erythromycin, gentamicin, imipenem, netilmicin, penicillin, rifampicin, trimethoprim and vancomycin. Norfloxacin and ofloxacin have less activity, and *Listeria* spp. are resistant to the cephalosporins, phosphomycin and polymyxin (MacGowan, 1990; Riviera *et al.*, 1993).

*Listeria monocytogenes* isolated from 9 of the 670 women with bad obstetric history were found to be sensitive to tetracycline, methicillin and erythromycin followed by streptomycin, ampicillin, chloramphenicol and penicillin. The isolates were found to be highly resistant to sulphonamides

(Bhujawala and Hingori, 1975). Isolates recovered from cases of *Listeriosis* in sheep and goats were found to be sensitive to terramycin, chloramphenicol, streptomycin and resistant to erythromycin, penicillin and sulphadiazine (Phadke *et al.*, 1979).

For effective treatment and control of *Listeriosis* both in animals and humans, prompt diagnosis is must. In the light of above review of the literature, a systematic study aimed at screening the abortion samples from sheep for the presence of *Listeria* spp. and finding the antibiogram of the isolates of *Listeria* spp. obtained was planned and executed.

## **CHAPTER – 3**

### **MATERIALS AND METHODS**

#### **3.1 Prevalence**

Cases of abortions and stillbirths in sheep in organized and unorganized farms of Kashmir valley were selected for the present study. The organized farms under the study included Sheep Breeding Farm Dachigam, Kralpathri., Zawoor, Gaobal and Sheep Research Station , Shuhama. The samples from unorganized farms were collected from local breeders from different districts of the valley viz. Pulwama, Budgam,

Anantnag, Ganderbal, Baramulla and Srinagar

### **3.2 Sampling**

Heart blood, liver, stomach contents, lungs, brain and kidneys of the aborted/ stillbirth lambs were collected aseptically in ice packs and brought to Veterinary Public Health laboratory immediately. The samples were processed for isolation of *Listeria* spp. within six hours of collection. The suspected brain tissues were also kept at 4°C for 2-4 weeks and then processed for isolation of *Listeria* spp.

### **3.3 Isolation**

25 g of each sample were inoculated into 100 ml of Brain Heart Infusion Broth and incubated at 37°C for 24-48 hrs. A loopful of culture was further streaked on *Listeria* selective agar (Hi media) and incubated at 37°C for 48 hours. Greenish -yellow colonies typical of *Listeria* spp were considered positive. These colonies were also streaked on PALCAM agar and incubated at 37°C for 48 hours for confirmation. Greyish colonies with black zone of aesculin hydrolysis were considered to be confirmatory for *Listeria monocytogenes*.

### **3.4 Biochemical tests**

#### **3.4.1 Catalase test**

The Catalase activity of the *Listeria* isolates was done as per the

method Cruickshank (1965). A fresh *Listeria* culture was mixed with a drop of 3% H<sub>2</sub>O<sub>2</sub> on a clean glass slide for demonstration of gas bubbles in positive cases.

### **3.4.2 Oxidase test**

*Listeria* isolates were tested for their oxidase activity as per the method of Cruickshank (1965). *Listeria* colony with positive aesculin hydrolysis on PALCAM was rubbed onto a filter paper strip soaked in freshly prepared solution (1%) of Redox dye (Tetramethyl-p-phenylene-diamine). The development of deep purple colour within 5-10 seconds was taken as a strong oxidase-positive reaction while no colouration even after 60 seconds was taken as oxidase-negative.

### **3.4.3 Methyl Red test**

The test was carried out as per the method of Cruickshank (1965). Each of the *Listeria* isolates was inoculated into a tube containing 0.5 ml sterile glucose phosphate broth and incubated at 37°C overnight. Subsequently, the tube was added with one drop of methyl red (MR) solution and observed for development of bright red colour as an indication of a positive MR test.

### **3.4.4 Voges-Proskauer test**

The Voges-Proskauer (VP) test was carried out as per the method of Cruickshank (1965). Each of the *Listeria* isolates was inoculated into 2 ml of sterile glucose phosphate peptone water and incubated at 37°C for 24 h. The tube was then added with 0.2 ml of 40% potassium hydroxide solution followed by 0.6 ml of 5%  $\alpha$ -naphthol solution and shaken vigorously. The cotton plug was removed and the tube left at room temperature for 1 h. The development of pink red colour in the solution was taken as VP- positive test.

#### **3.4.5 Nitrate reduction test**

All the *Listeria* isolates showing positive MR and VP reaction were subjected to nitrate reduction test as per the method of Cruickshank (1965). 5 ml of sterile nitrate broth was inoculated with a heavy growth of *Listeria* isolates and incubated at 37°C for 24 h. Subsequently, one drop of sulphanalic acid and the  $\alpha$ -naphthylamine reagent each was added to the culture in broth. The development of red colour within one minute was taken as positive reaction. The tubes not showing red colour within five minutes were treated with zinc powder (5 mg/ml of culture) and allowed to stand for 5 min. In such cases, the development of red colour indicated the presence of nitrate in the medium, as it was not reduced to nitrite by the test

organism

### **3.4.6 Gelatin liquefaction and Urea hydrolysis**

All the *Listeriae* isolates were tested for Gelatin liquefaction and urea hydrolysis as the method described by Cowan and Steel (1993).

### **3.4.7 Sugar fermentation tests**

All the catalase-positive, oxidase-negative, MR and VP-positive and nitrate- negative *Listeria* isolates were tested for xylose, rhamnose and  $\alpha$ -methyl D-mannopyranoside fermentation patterns as per the method of Cowan and Steel (1993). The change of colour of bromocresol purple (0.2%) from purple to yellow was considered as an indication of carbohydrate fermentation.

### **3.5 Motility test**

The *Listeria* isolate colonies were grown in Brain Heart Infusion broth for 18 hrs at 22°C. The isolates were observed for characteristic tumbling movement by hanging drop method. Further Motility was tested by inoculation of *Listeria monocytogenes* in semisolid agar and kept for 24 hr 22°C.

### **3.6 Hemolysis on sheep blood agar**

All the *Listeria* isolates were tested for the type and the degree of

hemolysis on Sheep Blood Agar (SBA). Following inoculation into SBA, the plates were incubated at 37°C for 24 hrs and examined for hemolytic zones around the colonies. A narrow zone of beta hemolysis was considered characteristic of *Listeria monocytogenes*.

### **3.7 Anton test/ demonstration of monocytosis in rabbits**

Approximately one million actively motile *Listeria monocytogenes* were instilled into the left eye of two adult healthy Newzealand white rabbits. The rabbits were observed for kerato conjunctivitis upto 8 days following instillation. Further the monocytes were counted upto 120 hrs post inoculation.

### **3.8 Experimental induction of abortion in rabbits**

One ml of  $10^7$  actively growing *Listeria monocytogenes* in NSS was inoculated intra peritoneally into each of the two pregnant does on 20<sup>th</sup> day of gestation period. The does were observed for seven days for signs of abortion. The pregnancy was diagnosed by ultrasonography (Plate 1).

### **3.9 Amplification of Listeriolysin O (*hyl A*) gene**

All the eight isolates isolated from the cases of abortions were screened for their virulent and non virulent status by verifying the presence or absence of *Listeriolysin O (hyl A)* gene. The amplification of the gene

was carried out by following the protocol of Noterman *et al.*, 1991a.

Isolates of *Listeria monocytogenes* were grown overnight in Brain Heart Infusion Broth at 37°C. Out of this broth culture, 0.5ml culture was transformed to micro-centrifuge tube and centrifuged at 12000 rpm for 10 min. The supernatant was decant off and pellet was resuspended in 500µl of phosphate buffer saline solution (PBSS) and re-centrifuged at 12000 rpm for 10 min. The recovered pellet was resuspended in 100µl of sterilized DNase and RNase free milliQ water. The pellet was suspended in the added water by vortexing and the lysis of the bacteria performed by boiling the tube for 10min followed by snap chilling in crushed ice. 2.5 µl of the obtained lysate was utilized as template DNA in amplification reaction volume of 25 µl. The details of the primers used are presented in Table-1.

**Table-1. Details of the primers used for amplification of *hyl A* gene**

Target gene	Primer sequence	Product size (bp)	G+C content
<i>hyl A</i> Forward	5'-GCAGTTGCAAGCGCTTGG AGTGAA-3'	456	54%
Reverse	GCAACGTATCCTCCAGAGTG ATCG-3'		54%

The reaction was performed in 0.2µl PCR tube containing reaction

mixture as given in Table 2.

**Table 2. Reaction mixture contents used in PCR tube**

S. No.	Contents	Volume	Concentration
1.	Template DNA	2.5 $\mu$ l	25 ng
2.	10X PCR buffer (Fermentas)	2.5 $\mu$ l	10 X
3.	10mMdNTP (Fermentas)	0.5 $\mu$ l	10mM
4.	25mM MgCl <sub>2</sub> (Fermentas )	2.0 $\mu$ l	25mM
5.	Primers (forward + reverse)	3.0 $\mu$ l	10 ng/ $\mu$ l
6.	Taq DNA polymerase	0.1 $\mu$ l	5 u/ $\mu$ l
7.	Distilled water	14.4 $\mu$ l	-
8.	Total	25.0 $\mu$ l	-

The reaction conditions for amplifying the *hyl* A gene were employed as per Notterman *et al.* (1991a) given in Table 3.

**Table-3. Reaction conditions used for amplifying the *hyl* A gene**

S. No.	Step	Temperature (°C)	Time
1.	Initial denaturation	94	2 min.
2.	Second denaturation	94	15 sec.
3.	Annealing	57	30 sec.
4.	Cyclic extension	72	1 min. 30 sec.

5.	Final extension	72	5 min.
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### 3.10 Agarose Gel Electrophoresis

The PCR products were analyzed by submersive agarose gel electrophoresis to resolve the amplified DNA fragments of the target gene. The agarose suspension (1.5%) was prepared by mixing molecular biology grade agarose (Sigma Aldrich) in 0.5x TBE buffer and heated to dissolve it completely. The molten agarose was cooled to 50°C. Ethidium bromide (SRL) was added to a final concentration of 0.5µg/ml before pouring in an evenly levelled gel tray already fitted with gel comb and open ends sealed with adhesive tape. The gel tray was left undisturbed for about an hour to allow proper gelling of agarose in buffer. The gel tray was placed in submersive electrophoresis tank (Atto Japan) with the wells' side towards the cathode end and sufficient quantity of electrophoresis buffer (TBE, 0.5x) was poured to submerge the gel.

2.5 µl of PCR product from each sample was mixed with 6x bromophenol blue loading dye (2µl) and loaded into each well along with a DNA ladder (Fermentas) as marker in a separate well. The electrophoretic tank was covered with a lid and connected to electrophoresis power supply

unit (Genei, Bangalore). The electrophoresis was carried out at 60 volts until the dye front approached the anode end. The resolution of bands in the gel was visualized by a UV transilluminator and digitally recorded by gel documentation system (ULTRA LAMP).

### **3.11 Antibiogram of *Listeria* isolate**

All the *Listeria* isolates were subjected to antibiotic sensitivity by disc diffusion method as described by Bauer *et al.* (1966). The antibiotic discs used were gentamicin, doxycycline, tetracycline, ampicillin, penicillin G, amoxycillin, cephatoxime, amikacin, lemfloxacin, erythromycin, chloromphenicol, ciprofloxacin, nalidixic acid and cotrimaxazole.

## **CHAPTER – 4**

### **EXPERIMENTAL FINDINGS**

#### **4.1 Prevalence**

Fifty two cases of abortions/stillbirths in ewes of organized and unorganized farms of Kashmir valley were tested for isolation and identification of *Listeria monocytogenes*. The organism was isolated from eight cases with a prevalence of 15.38 per cent. The prevalence was higher (Table 4) in the organized (18.51%) than in the unorganized farms

(12.00%).

Out of 12 cases of abortions/stillbirths at Sheep Breeding Farm Dachigam, *Listeria monocytogenes* was isolated from three with a prevalence of 25.00 per cent and of the 4 cases of abortions/stillbirths at Sheep Breeding Farm Gaobal, *Listeria monocytogenes* was recovered from one with a prevalence of 25.00 per cent. The prevalence, however, was low (11.11%) at Sheep Research Station, Shuhama where only one out of 9 cases revealed *Listeria monocytogenes*. With only one case of abortion/stillbirth each reported from Sheep Breeding Farms, Kralpathri and Zawoor, *Listeria monocytogenes* could not be isolated from any of these cases.

**Table-4. Prevalence of *Listeria monocytogenes* in organized Sheep Breeding Farms of Kashmir**

S. No.	Name of the farm	No. of abortions/ stillbirths	Cases positive for <i>Listeria</i> spp.	Percent positive for <i>Listeria</i> spp.
1.	Sheep Breeding Farm, Dachigam	12	3	25.00
2.	Sheep Breeding Farm, Gaobal	4	1	25.00
3.	Sheep Breeding Farm, Kralpathri	1	0	0.00

4.	Sheep Breeding Farm, Zawoora	1	0	0.00
5.	Sheep Research Station, Shuhama	9	1	11.11

In the unorganized farms, district Budgam revealed the highest prevalence (28.57%) with *Listeria monocytogenes* having been isolated from two out of seven cases of abortion/stillbirths. Out of seven cases of abortions/stillbirths in district Pulwama, *Listeria monocytogenes* was recovered from one with a prevalence of 14.28 per cent. However, with three cases of abortions/stillbirths each reported from district Ganderbal and Baramulla, the organism was not recovered from any of these cases. *Listeria monocytogenes* was also not isolated from any of the four cases of abortions/stillbirths from district Anantnag and also from a lone case of abortion/ stillbirth in district Srinagar (Table-5).

**Table-5. Prevalence of *Listeria monocytogenes* in unorganized Sheep Breeding Farms of different Districts of Kashmir**

S. No.	Name of the farm	No. of abortions/ stillbirths	Cases positive	Per cent positive
1.	Unorganized Sheep Farms, District Pulwama	7	1	14.28

2.	Unorganized Sheep Farms, District Budgam	7	2	28.57
3.	Unorganized Sheep Farms, District Baramulla	3	0	0.00
4.	Unorganized Sheep Farms, District Anantnag	4	0	0.00
5.	Unorganized Sheep Farms, District Ganderbal	3	0	0.00
6.	Unorganized Sheep Farms, District Srinagar	1	0	0.00

None of the aborted ewes (Plate-2a) exhibited any characteristic clinical signs of *Listeriosis*. Half of the still-born lambs positive for *listeriosis* were mummified, while others were oedematous and had clear or blood tinged fluid in their abdominal and thoracic cavities. Haemorrhages in the sub cutis, skin, lungs, epicardium and in the kidneys and the mesenteric lymph nodes of aborted/stillborn foetuses were observed. Yellowish white necrotic foci on the lobes of the livers were seen in 37.5 per cent of the still-born lambs (Plate 2b), while others presented focal or multifocal haemorrhages on the surface of the livers. The livers of foetuses had rounded borders, were friable in consistency and yellowish brown in colour (Plate 3a). Moderate to severe congestion of brain was also observed in 6 of the 8 positive cases (Plate 3b). In some cases, however, the

medullary meninges were thickened by a gelatinous oedema. Most of the brains had an increased amount of cerebro-spinal fluid, being cloudy in some cases (Plate 3b). Deformity of limbs was also noticed in one of the aborted lambs. Out of the eight isolates of *Listeria monocytogenes* recovered, four were from only brain tissues, two from brain and livers and one each from liver and stomach.

#### **4.2 Microbiological isolation:**

On *Listeria* selective agar (Hi media), *Listeria monocytogenes* produced typical smooth greenish-yellow colonies in 48 hours of incubation at 37°C (Plate 4a). On PALCAM agar, the organism produced smooth, small greyish colonies with blackening of the surrounding media representing aesculin hydrolysis *i.e.* the characteristic of *Listeria monocytogenes* (Plate 4b). When these colonies were subjected to Gram's staining, typical gram positive cocco-bacillary rods were observed (4a). These rods appeared in singles, doubles, V shapes, parallels representing a palisade arrangement (Plate 5a). Typical tumbling motility was observed in 18 hr. old brain heart infusion broth incubated at 22°C. Motility was also demonstrated in semisolid agar slants by stab culture in 18-24 hr at 22 degree Celsius as umbrella like growth (Plate 8a).

All the *Listeria monocytogenes* isolates subjected to different

biochemical tests gave a positive reaction for catalase, methyl-red and Voges-Proskauer and a negative reaction for oxidase, nitrate reduction, gelatin liquefaction and urea hydrolysis, besides fermenting rhamnose sugar (Table 6).

**Table-6. Results of biochemical tests of *Listeria monocytogenes* isolates**

S. No.	Biochemical test	Result
1.	Catalase test	+ ve
2.	Oxidase test	-ve
3.	Methyl red test	+ve
4.	Voges-Proskauer test	+ve
5.	Nitrate reduction test	-ve
6.	Gelatin liquefaction	-ve
7.	Urea hydrolysis	-ve
8.	Mannitol fermentation	-ve
9.	Rhamnose fermentation	+ve
10.	Xylose fermentation	-ve

#### **4.3 Hemolysis on blood agar**

All the isolates of *Listeria monocytogenes* were tested for their hemolytic activity in 10 per cent sheep blood agar. The isolates showed narrow zones of beta hemolysis, a characteristic feature of *Listeria monocytogenes* (Plate 5b).

#### **4.4 Animal inoculation tests**

##### **4.4.1 Anton test/demonstration of monocytosis in rabbits**

Two drops of broth culture containing one million live *Listeria*

*monocytogenes* when instilled into left eyes of two rabbits, resulted in kerato-conjunctivitis (Plate 6a) which was mild starting from 4<sup>th</sup> day and becoming more pronounced on 6<sup>th</sup> day post inoculation. The blood picture presented leukocytosis (Plate 6b) with monocytes increasing upto 15 per cent by 48 hr post inoculation and becoming normal by 120 hr post inoculation (Fig. 1).

#### **4.4.2 Experimental induction of abortion in rabbits**

Intraperitoneal inoculation of  $10^7$  viable *Listeria monocytogenes* contained in 1ml of NSS into two pregnant does on 20<sup>th</sup> day of gestation period, resulted in abortion within 48 hr post inoculation. The rectal temperature increased by 2°C from day second post inoculation and subsided on the day of abortion. The does showed marked vaginal discharges starting from 24 hr post inoculation up to 3 days following abortion. The conceptus was expelled intact. The amniotic fluid was blood tinged and dark red in colour. The thick, blood tinged vaginal discharge collected aseptically revealed *Listeria monocytogenes* on cultural examination. The organism was also isolated from organs of the aborted foetuses.

#### **4.5 Polymerase chain reaction**

All the *Listeria monocytogenes* isolates obtained were subjected to

polymerase chain reaction for the detection of virulence associated gene (*hyl A*) responsible for the production of a hemolysin called *Listeriolysin O*. All the isolates were positive for *hyl A* gene and produced strong amplification products. Amplification revealed a band at approximately 456 bp which was in agreement with the expected size (Plate 7).

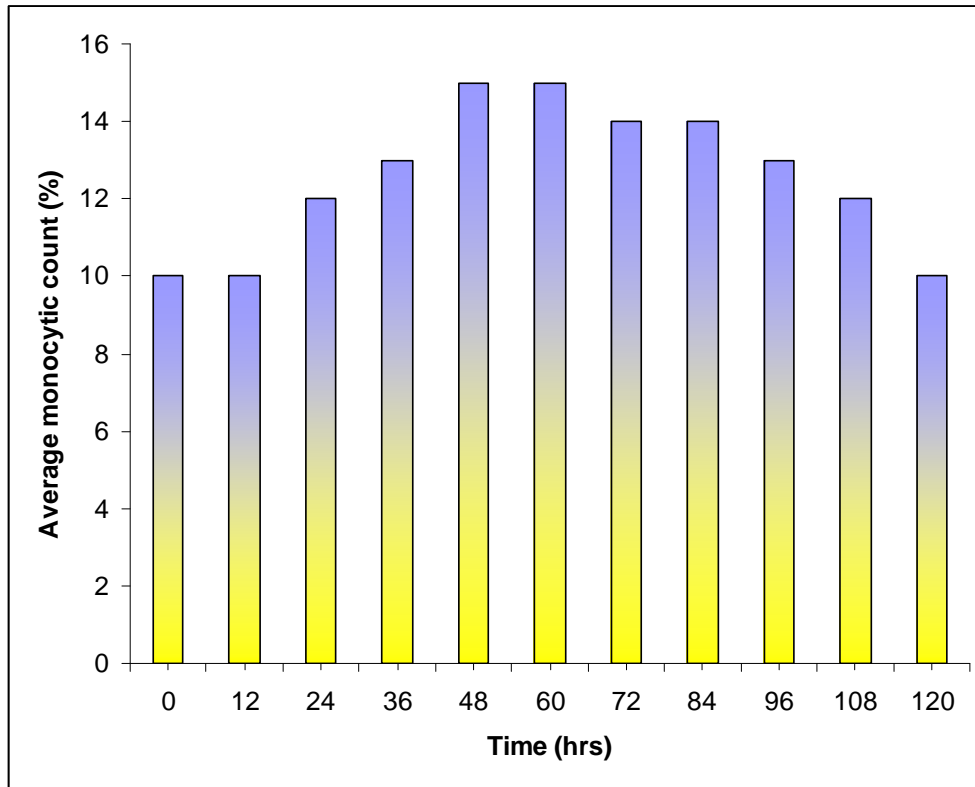
#### 4.6 Antibiogram of *Listeria monocytogenes* isolates

The eight isolates of *Listeria monocytogenes* were subjected to paper disc diffusion test for in- vitro sensitivity test (Bauer *et al.*, 1966). The isolates showed variable results against different antimicrobials. They were 100, 87.5, 75, 75 and 62.5 per cent sensitive to gentamicin, doxycycline, tetracycline, ampicillin and penicillin G respectively, highly resistant against co-trimoxazole, nalidixic acid, ciprofloxacin, chloramphenicol, erythromycin, lemfloxacin and moderately sensitive against amikacin, cephatoxime and amoxicillin (Table 7; Plate 8b).

**Table-7. Antibiotic sensitivity pattern of *Listeria monocytogenes* to different antibiotics**

S. No.	Antimicrobial agent	Sensitive	Moderately sensitive	Resistant
1.	Gentamicin (10 mcg)	8/8 (100%)	-	-
2.	Doxycycline (30 mcg)	7/8 (87.5%)	1/8 (12.5%)	-
3.	Tetracycline (30 mcg)	6/8 (75%)	1/8 (12.5%)	1/8 (12.5%)

4.	Ampicillin (10 mcg)	6/8 (75%)	1/8 (12.5%)	1/8 (12.5%)
5.	Penicillin G (10 units)	5/8 (62.5%)	2/8 (25%)	1/8 (12.5%)
6.	Amoxicillin (30 mcg)	4/8 (50%)	2/8 (25%)	2/8 (25%)
7.	Cephatoxime (30 mcg)	4/8 (50%)	2/8 (25%)	2/8 (25%)
8.	Amikacin (30 mcg)	2/8 (25%)	4/8 (50%)	2/8 (25%)
9.	Lemofloxacin (10 mcg)	1/8 (12.5%)	2/8 (25%)	5/8 (62.5%)
10.	Erythromycin (15 mcg)	1/8 (12.5%)	2/8 (25%)	5/8 (62.5%)
11.	Chloramphenicol (30 mcg)	1/8 (12.5%)	-	7/8 (87.5%)
12.	Ciprofloxacin (5 mcg)	-	1/8 (12.5%)	7/8 (87.5%)
13.	Nalidixic acid (30 mcg)	-	1/8 (12.5%)	7/8 (87.5%)
14.	Co-trimoxazole (1.2 mcg)	-	-	8/8 (100%)



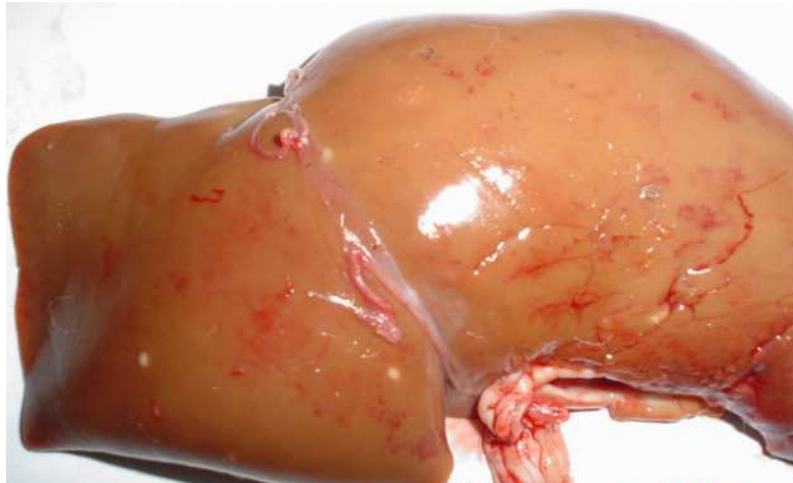
**Fig.1. Average monocytic count in rabbits following intra-ocular inoculation of *Listeria monocytogenes***



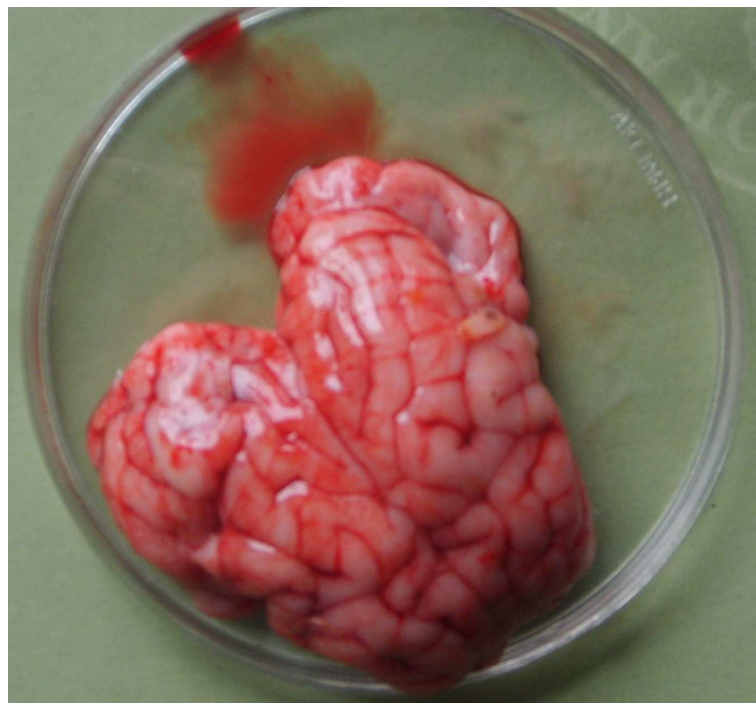
**Plate-2a. An ewe giving birth to an aborted fetus**



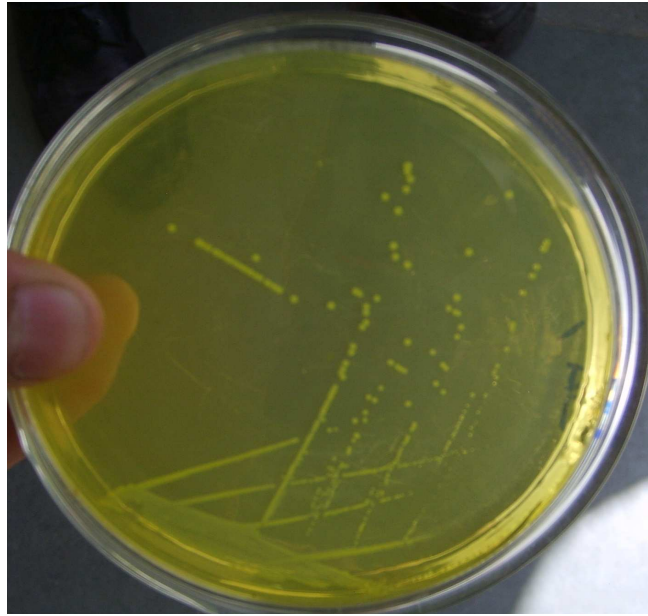
**Plate 2b. A stillborn lamb**



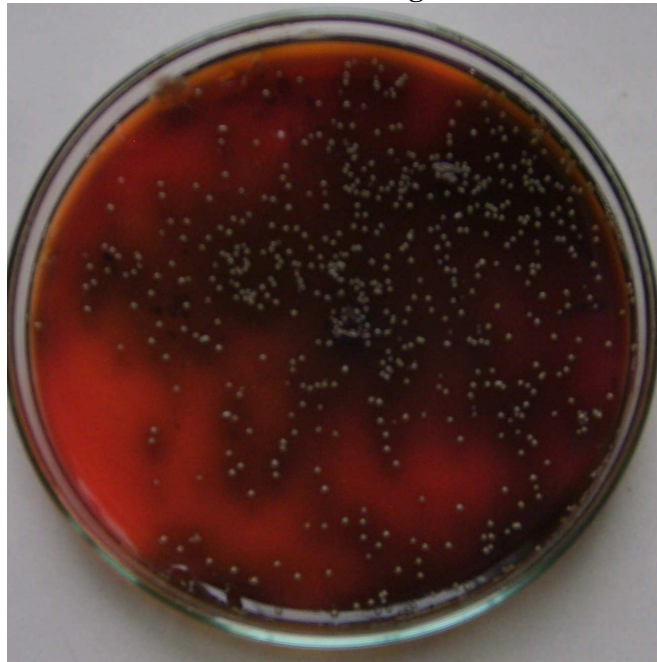
**Plate 3a. Focal haemorrhages and necrotic foci on the liver of stillborn lamb**



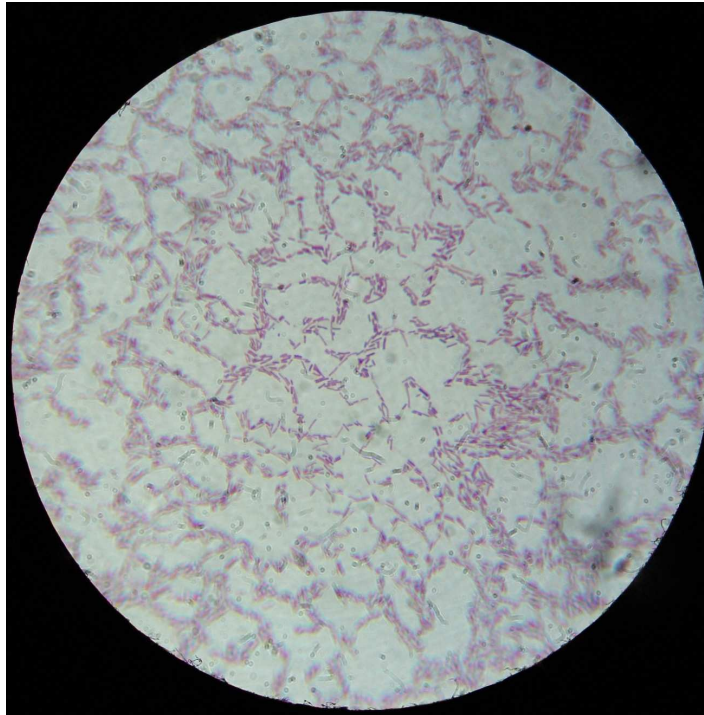
**Plate 3b. Brain of a stillborn lamb showing congestion and haemorrhages**



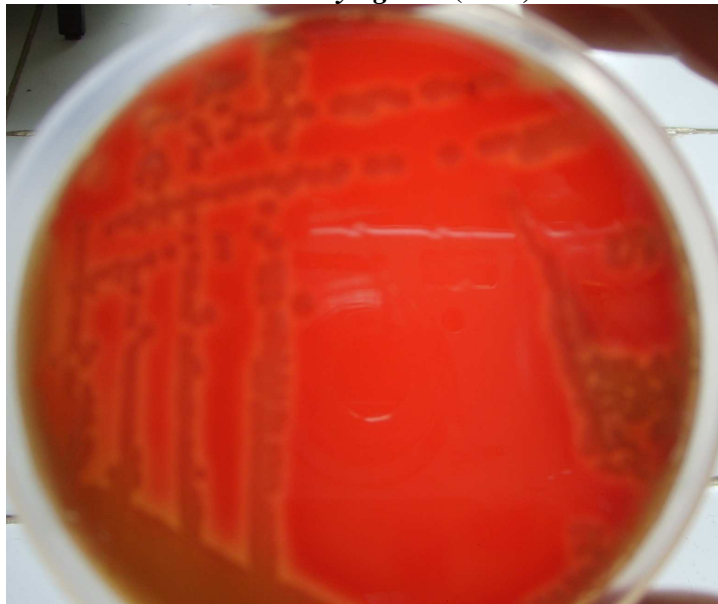
**Plate 4a. Greenish yellow colonies of *Listeria* spp. on Listeria selective agar**



**Plate 4b. Grayish Colonies of *Listeria monocytogenes* on PALCAM Agar with blackening of surrounding media**



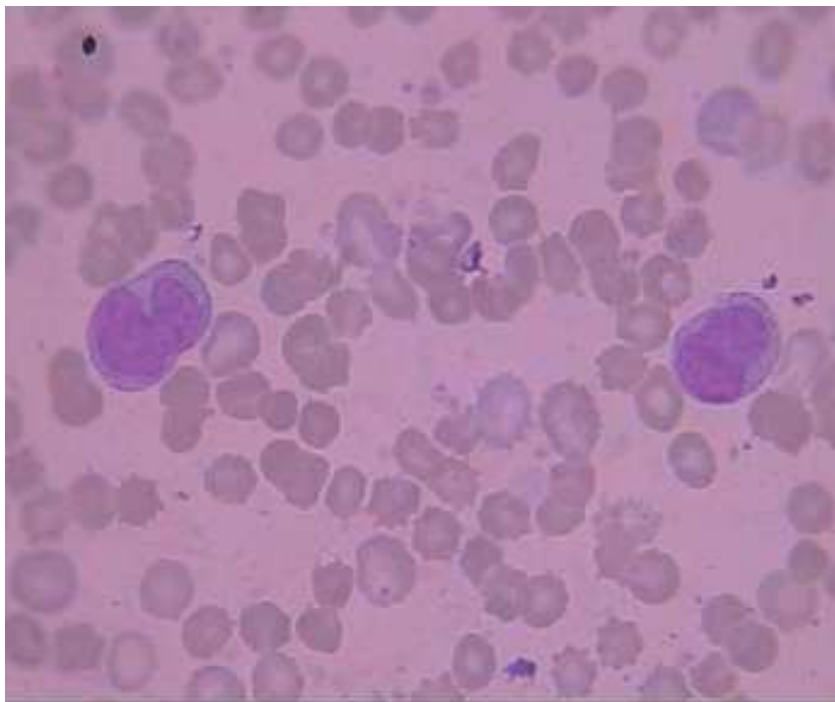
**Plate 5a. Gram positive cocco-bacillary rods of *Listeria monocytogenes* (100x)**



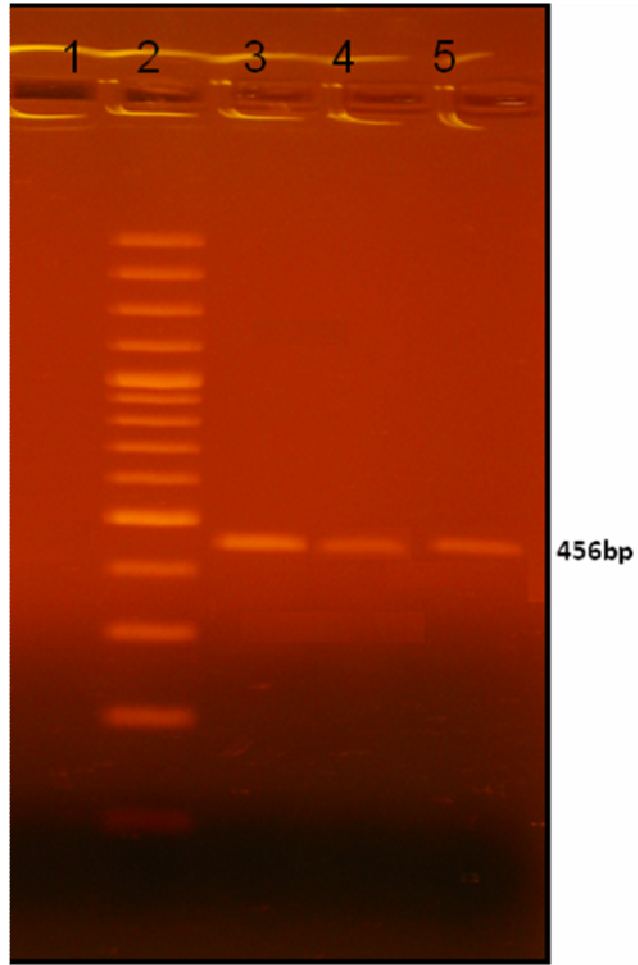
**Plate 5b. Sheep Blood agar plate showing zones of hemolysis around the colonies**



**Plate 6a. Anton test depicting kerato-conjunctivitis due to intra-ocular inoculation of live *Listeria monocytogenes* culture in rabbit (right) against the control (left)**



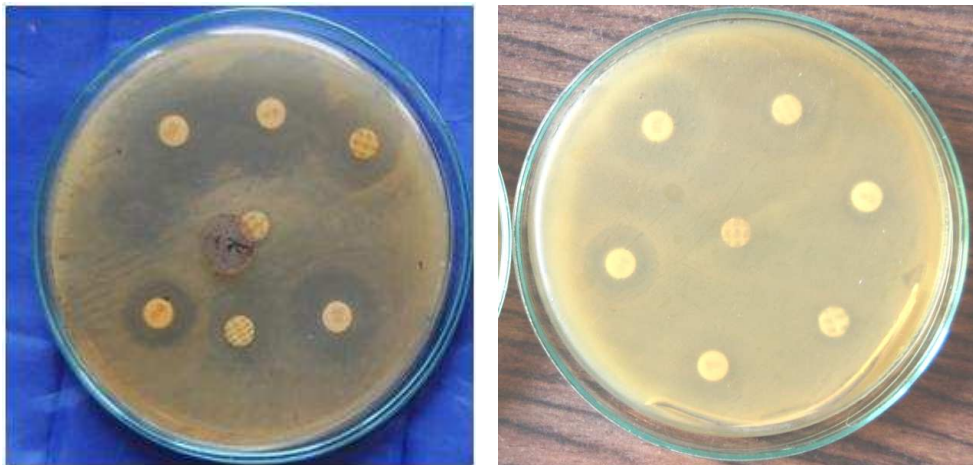
**Plate 6b. Monocytosis in rabbit post intra-ocular instillation**



**Pate 7.** Bands revealing amplified products of *hyl A* gene of *Listeria monocytogenes* (1-negative control, 2-DNA ladder, 3,4,5- PCR products)



**Plate 8a. Motility in semi-solid agar (Umbrella shaped growth of *Listeria monocytogenes*)**



**Plate 8b. Antibiotic sensitivity pattern of *Listeria monocytogenes***

## CHAPTER – 5

### DISCUSSION

Reproductive disorders in animals as a result of infectious diseases are common worldwide. These infections constitute for major economic losses in the animal sector by way of deaths of the newborn and also due to the stressed conditions of the dams leading to under productivity. Besides, most of these infections are of zoonotic importance and, therefore, put the human population to a constant threat. Although an uncommon cause of illness in the general population, *listeriosis* in man is often severe with mortality rates reaching up to 50 per cent. The disease is common in temperate regions throughout the world and, therefore, the organism's sustainability is assured in places like Himachal Pradesh, Jammu & Kashmir and other temperate regions of the country. Not much is known, however, about the role of listeric infections in causation of reproductive disorders in man and animals especially in the Indian sub-continent as most of the natural cases go unnoticed or undetected due to lack of proper laboratory diagnostic facilities.

*Listeriosis* was first reported from Jammu & Kashmir by Vishwanathan and Uppal (1981) in cases of abortions in organised sheep farms. Since its first reporting, no work was carried out to ascertain the

prevalence of this disease in the state till 2005 when Willayat *et al.* (2005) reported 26.99 per cent cases of abortions in an organised sheep farm of Kashmir valley. In the present investigation an attempt was made to study the prevalence of the disease in both organised and unorganised sheep farms and identify the causal agent by more recent techniques like PCR. The present study was confined to the recovery of *Listeria* species from cases of abortions and still births in pregnant ewes based on the reports of the earlier workers. An overall prevalence of 15.38 per cent was recorded in cases of abortions and still-births in these farms. The lower prevalence observed in the present study could be due to the better hygienic conditions having been followed subsequent to the reporting by previous workers (Willayat *et al.*, 2005). Higher prevalence was recorded in the organised farms compared to the unorganised sector which could partly be due to more confinement of the livestock in the winter months in the organised farms leading to a more stressed condition. As a general practice, animals are let loose in the unorganised farms even in the winter months. Variable reports are available on the prevalence of *Listeriosis* in sheep. Broadbent (1972) recorded an abortion rate of 20 per cent due to *Listeria monocytogenes* from 23 Victorian flocks in Australia. Similar findings were also recorded by Vishwanathan and Uppal (1981) who recorded a prevalence of 19.8 per cent from cases of abortions in J&K state. However,

Pasa *et al.* (2003) reported a higher prevalence of 35 per cent in sheep from France. A lower prevalence rate has also been reported by some workers (Nigam *et al.*, 1996). In the present study, higher prevalence of 25 per cent each was recorded in sheep breeding farms, Dachigam and Gaobal compared to Sheep Research Station, Shuhama where the prevalence was as low as 11.11 per cent. *Listeria monocytogenes* could not be isolated from some organised farms possibly because of very less number of samples available (Table 4). The variability in the prevalence rates could be due to the variability in the local sanitary and hygienic conditions in these farms. Similar justifications may hold true for the variability of the prevalence rates in the unorganised farms of the valley. Kashmir valley, as such, seems to be no exception to the sustenance of *Listeria monocytogenes* as reported elsewhere with similar topography and agro-climatic conditions. None of the aborted ewes presented any clinical manifestations of *listeriosis* prior to or following abortions/still-births which is in agreement with the findings of other workers (Kapoor and Sadna, 1974; Kennedy and Miller, 1993).

Half of the still-born lambs positive for *listeriosis* were mummified, while others were oedematous and had clear or blood tinged fluid in their abdominal and thoracic cavities. Similar findings were observed by McDonald (1967). Jeleff and Djurov (1969), while studying 24

spontaneously aborted ovine foetuses, from which *Listeria monocytogenes* was isolated, noticed that subcutaneous oedema was most pronounced in area of abdominal wall and head. They found 30-100 ml of blood tinged fluid in abdominal and thoracic cavities, also haemorrhages on the pleura, epicardium and liver of many foetuses. Similar findings have also been observed by others (Jeleff *et al.*, 1964; Ladds, 1970; Njuko *et al.*, 1972). In the present study, haemorrhages in the sub cutis, skin, lungs, epicardium and in the kidneys and the mesenteric lymph nodes of aborted/stillborn foetuses were also observed. Yellowish white necrotic foci on the lobes of the livers were seen in 37.5 per cent of the still-born lambs (Plate 3a), while others presented focal or multifocal haemorrhages on the surface of the livers. Jeleff and Djurov (1969) found miliary and sub-miliary grey white foci on the livers of aborted or still-born lambs in the listeric infections. Focal necrosis was observed in livers of 75 per cent of *Listeria* infected lambs by Dennis (1966). The livers of foetuses, observed in the present study, had rounded borders, were friable in consistency and were yellowish brown in colour. This is in accordance with the observations made by previous workers (Jeleff *et al.*, 1964; Ladds, 1970). Moderate to severe congestion of brain was also observed in 6 of the 8 positive cases (Plate 3b). In some cases, however, the medullary meninges were thickened by a gelatinous oedema. Most of the brains had an increased amount of cerebro-

spinal fluid, being cloudy in some cases. These findings are in agreement with the findings of Gray and Killinger (1966) and Chand and Sadna (1999). Abomasal ulcers, subcutaneous oedema with clear or blood tinged fluid in the abdominal and thoracic cavities have also been reported by some workers (Ladds, 1970; Njoku *et al.*, 1972). In the present study, some still-born lambs also showed deformities of limbs as observed by previous workers (Willayat *et al.*, 2005). But for being time consuming and laborious, cultural methods, isolation and differentiation of pathogenic from non-pathogenic *Listeriae* remain the most authentic methods of diagnosis at the laboratory level. The pathogenic potential of *Listeria* isolates have been assessed by *in-vitro* pathogenesis tests like beta-hemolysis on sheep or horse blood agar (Schonberg *et al.*, 1989), Christie, Atkins, Munch-Peterson (CAMP) test (Mckellar,1994), plating on Listeria agar according to Ottaviani and Agosti (ALOA) (Ottaviani *et al.*, 1997; Reissbrodt, 2004), and phosphatidyl-inositol specific phospholipase C (PI-PLC) assay (Notermans *et al.*,1991b). However, confirmation of *Listeria* isolates by *in-vivo* methods like chick embryo, mouse and rabbit inoculation tests is most reliable for linking these isolates to the real cases of *listeriosis*. In the present study, *Listeria monocytogenes* produced typical smooth greenish yellow colonies on *Listeria* selective agar within 48 hr at 37°C (Plate 4a). The cultural confirmation of the organism was, however, made in

PALCAM agar where greyish colonies with blackening of the surrounding media representing aesculin hydrolysis, was observed in 48 hr at 37°C (Plate 4b). The organism showed little or no motility in broth cultures at 37 °C in 18-24 hr conforming to the findings of the earlier workers (Gray and Killingher, 1966; Seeliger and Jones, 1986). However, a characteristic tumbling motility, as demonstrated by previous workers (Farber and Peterkin, 1991), was observed in these broth cultures at 22°C in 18-24 hr. Although relatively inactive biochemically, *Listeria monocytogenes* produces catalase and is positive for Voges-Proskauer and aesculin hydrolysis reactions. The organism gives a negative reaction for indole, oxidase, nitrate reduction and gelatin liquefaction and fails to hydrolyse urea. In the present study, the isolates of *Listeria monocytogenes* demonstrated biochemical reactions similar to those reported by earlier workers (Rocourt *et al.*, 1983).

Demonstration of beta haemolysis in sheep/ horse blood agar has been used as a tool for differentiating pathogenic *Listeriae* from non-pathogenic ones (Courteio, 1991). This is attributed to the presence of a haemolysin in *Listeria monocytogenes*, generally referred to as the *Listeriolysin O* which facilitates the organism to escape the intracellular killing within the macrophages thereby allowing it to multiply and spread

between cells. The importance of *Listeriolysin O* as a virulence factor has been clearly established (Gaillard *et al.*, 1986; Geoffroy *et al.*, 1987). The spontaneous loss of haemolysin production has been observed to result in the loss of virulence (Hof, 1984). However, virulence has not been found to be directly related with the level of *Listeriolysin O* production (Farber and Peterkin, 1991). All the isolates in the present study produced marked degree of beta haemolysis on 10 per cent sheep blood agar plates in 24-36 hr (Plate 5b ) depicting the extent of their pathogenicity in the host species.

Further confirmation of *Listeria monocytogenes* as the causative agent of abortion/stillbirths was done by animal inoculation tests. The 18 hr old broth culture produced kerato-conjunctivitis and monocytosis (Plate 6a and 6b) in healthy rabbits within 2- 6 days by intra-ocular route. Similar observations on experimental inoculation in rabbits have been made previously (Dutta and Malik, 1978; Radostitis *et al.*, 1994). Some workers have linked the monocytosis producing factor, extracted from the cell wall of *Listeria monocytogenes*, to its virulence on the plea that all the virulent strains of the organism were able to produce monocytosis in rabbits (Galsworthy *et al.*, 1977). The crude cell wall extracts of *Listeria monocytogenes* have many biological functions particularly stimulating the

cell mediated immune response especially against the tumour cells but their exact role in immunological responses is yet unclear.

Inoculation of viable *Listeria monocytogenes* into two pregnant does on 20<sup>th</sup> day of gestation by intra-peritoneal route resulted in abortion within 48 hr post inoculation. The vaginal discharge was thick; blood tinged, and revealed *Listeria monocytogenes* on cultural examination. The organism was also isolated from organs of the aborted foetuses. These findings are in agreement with the findings of Gray *et al.* (1955). The virulence of *Listeria monocytogenes* has been correlated with pathogenicity in the laboratory animals including mice (Mainou-Fowler *et al.*, 1988). Some workers have used embryonated eggs for demonstrating the death of embryos within 72 hr following inoculation of *Listeria monocytogenes* through chorio-allantoic route (Terplan and Steinmeyer, 1989).

Polymerase Chain Reaction (PCR) is used as an additional tool for rapid, sensitive and specific detection of microorganisms from different sources. This facilitates exponential amplification of a pre-selected region of DNA and thus represents a highly specific and sensitive detection technique. Amplification of specific DNA sequences is possible from purified plasmid, chromosomal DNA and also crude DNA samples. Thus PCR has allowed direct identification of organisms from complex substrates

without prior purification (Olive, 1989; Williams *et al.*, 1990). PCR based assays for the detection of *Listeria monocytogenes* in food and environmental channels have been successfully used by many workers (Rossen *et al.*, 1991; Norton, 2002). The genes of different virulent markers have also been used for the species-specific identification of *Listeria monocytogenes*. Recently PCR and nested PCR for the species-specific identification of *Listeria monocytogenes* using primers against *hyl A* gene was standardised with a minimum detection level of 5 cells per reaction (Balamruga, 2002). Several virulence associated genes have been sequenced and used as PCR-targets for the detection of *Listeria monocytogenes* e.g. *Listeriolysin O (hyl A)* gene (Border *et al.*, 1990; Rossen *et al.*, 1991), the *Dth 18* gene (Wernars *et al.*, 1991), the *iap A* gene (Jaton *et al.*, 1992) and the *plc A* gene (Notermans *et al.*, 1991b). *Listeriolysin O* encoded by *hyl A* gene has been regarded as the most important virulence factor.

In the present study, all the isolates of *Listeria monocytogenes* were confirmed by amplification of the *hyl A* gene. The isolates produced a strong amplification of the target gene thereby confirming the isolates as *Listeria monocytogenes*. According to some workers, detection of only one virulence associated gene by PCR is not always sufficient to identify

*Listeria monocytogenes* and that PCR amplification of genes other than *hyl A* and *plcA* may not be adequate for the detection of virulent strains of *Listeria monocytogenes* (Nishibori *et al.*, 1995). In order to avoid the possible failure in the detection of virulent *Listeria monocytogenes*, a one step procedure, enabling the demonstration of three virulence-associated genes, *prf A*, *hyl A* and *plc B* simultaneously in a single PCR mixture, was developed for a large scale survey (Cooray *et al.*, 1994). Recently, multiplex PCR targeting virulence genes of *Listeria monocytogenes* (*plc A*, *prf A* and *hyl A* genes) have been employed (Rawool, 2004).

Isolates of *Listeria monocytogenes* under study were highly sensitive to gentamicin (100%), doxycycline (87.5%), tetracycline (75%), and ampicillin (75%). However, all the isolates showed a high degree of resistance towards co-trimoxazole, nalidixic acid, ciprofloxacin, chloramphenicol, erythromycin and lemfloxacin. Sensitivity to gentamicin and resistance to chloramphenicol and nalidixic acid has also been reported by Willayat *et al.* (2005) McGowan (1990) has proposed an *in-vivo* treatment of choice for *listeriosis* as the combination of ampicillin and an amino-glycoside viz. gentamicin. Isolates recovered from cases of *listeriosis* in sheep and goats by some workers (Phadke *et al.*, 1979) were sensitive to terramycin, chloramphenicol, streptomycin and resistant to

erythromycin, penicillin and sulphadiazine, while those recovered from the samples of milk have shown high degree of sensitivity to doxycycline, ampicillin and ciprofloxin. Some workers have reported resistance of *Listeria monocytogenes*, recovered from infertile cows, to nalidixic acid, polymixin B and sulphonamides (Srivastava *et al.*, 1985).

## CHAPTER – 6

### SUMMARY AND CONCLUSION

Prevalence of Listeric abortions in organized and unorganized sheep farms of Kashmir valley was studied during 2008-2009. The organized farms included; Sheep Breeding Farms, Dachigam, Kralpathri, Zawoora, Gaobal and Sheep Research Station, Shuhama. The unorganized farms comprised of the local sheep breeders from different districts of the valley.

In all, 52 cases of abortions/stillbirths were screened for isolation and identification of *Listeria* species. *Listeria monocytogenes* was the only species of Genus *Listeria* isolated from cases of abortions/stillbirths, under study. An overall prevalence of 15.38% of *Listeriosis* was recorded in these farms. The prevalence was higher in organized (18.51%) than in the un-organized farms (12.00%).

Half of the aborted/still-born lambs positive for *listeriosis*, were mummified while others were oedematous and had clear or blood tinged fluid in their abdominal and thoracic cavities. Haemorrhages in the sub cutis, skin, lungs, epicardium, kidneys and the mesenteric lymph nodes of aborted/stillborn foetuses were also observed. Yellowish white necrotic foci on the lobes of the livers were seen in 37.5 per cent of the still-born lambs,

while others presented focal or multifocal haemorrhages on the surface of the livers.

The isolates produced typical smooth greenish yellow colonies on Listeria selective agar within 48 hr at 37 degrees Celsius. The cultural confirmation of *Listeria monocytogenes* was made in PALCAM agar where greyish colonies with blackening of the surrounding media representing aesculin hydrolysis, was observed in 48 hr at 37 degrees Celsius. The organism showed little or no motility in broth cultures at 37 degrees Celsius in 18-24 hr. However, a characteristic tumbling motility was observed in these cultures at 22 degrees Celsius in 18-24 hr.

Out of a total of 8 isolates recovered, 4 were isolated from only brain tissues, 2 from both brain and livers and one each from liver and stomach contents. All the isolates under study produced marked degree of beta haemolysis on 10% sheep blood agar plates in 24-36 hr depicting the extent of their pathogenicity in the host species.

In the present study, all the isolates of *Listeria monocytogenes* were confirmed by amplification of the *hyl A* gene. The isolates produced a strong amplification of the target gene thereby confirming the organism as *Listeria monocytogenes*.

The 18 hr old broth culture produced kerato-conjunctivitis and

monocytosis in healthy rabbits within 2-6 days by intra-ocular route. Inoculation of viable *Listeria monocytogenes* by intra peritoneal route, into two pregnant does, on 20<sup>th</sup> day of gestation, resulted in abortion within 48 hr post inoculation. The vaginal discharge was thick; blood tinged, and revealed *Listeria monocytogenes* on cultural examination. The organism was also isolated from organs of the aborted foetuses.

All the isolates of *Listeria monocytogenes* under study, were sensitive to gentamicin, doxycycline, ampicillin, tetracycline and penicillin G in order and resistant to nalidixic acid, co-trimoxazole, ciprofloxacin, chloramphenicol, erythromycin and lomoloxacin.

## CONCLUSION

- An overall prevalence of 15.38 percent listeric abortions was recorded in sheep of Kashmir. The prevalence in organized farms was higher (18.51%) than the unorganized farms (12%).
- *Listeria monocytogenes* was the only species of the genus *Listeria* isolated from the cases of abortion/stillbirths.
- All the isolates were virulent in the host species as they produced marked degree of beta haemolysis on 10 per cent sheep blood agar plates.

- The organism produced kerato-conjunctivitis and monocytosis in healthy rabbits within 2- 6 days by intra-ocular route.
- Inoculation of  $10^7$  viable *Listeria monocytogenes* into two pregnant does on 20<sup>th</sup> day of gestation by intra-peritoneal route resulted in abortion within 48 hr post inoculation.
- All the isolates produced strong amplification of *hyl* A gene by polymerase chain reaction.
- The isolates were highly sensitive to gentamicin, doxycycline, tetracycline and ampicillin and resistant to co-trimoxazole, nalidixic acid, ciprofloxacin, chloramphenicol, erythromycin and lemfloxacin.

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## APPENDIX – I

### 1. BRAIN HEART INFUSION BROTH

<u>Ingradients</u>	<u>g/litre</u>
Peptic digest of animal tisssu	10.00
Calf brain infusion (solids)	12.50
Beef heart infusion (solids)	5.00
Dextrose	2.00
Sodium chloride	5.00
Disodium phosphate	2.50

Suspend 37 g of the medium into 1000ml distilled water. Heat to dissolve the medium completely. Sterilize by autoclaving at 15lb (121°C) for 15 minutes.

### 2. LISTERIA SELECTIVE AGAR (Part A)

<u>Ingradients</u>	<u>g/litre</u>
Casein enzymic hydrolysate	10.00
Peptic digest of animal tisse	10.00
Dextrose	1.00
Sodium chloride	5.00
Thiminium dichloride	0.001
Acriflavin hydrochloride	0.01
Nalidixic acid	0.004
Agar	13.00

### Part B

Potassium thiocyanate 37.50

Suspend 39.015 g of part A with 37.50 g of part B into 1000 ml distilled water. Heat to boiling to completely dissolve the medium. Sterilize by autoclaving at 15lb (121°C) for 15 minutes.

### 3. PALCAM AGAR

<u>Ingredients</u>	<u>g/litre</u>
Peptic digest of animal tissue	23.00
Starch	1.00
Sodium chloride	5.00
Mannitol	10.00
Ammonium ferric citrate	0.50
Dextrose	0.50
Lithium chloride	15.00
Phenol red	0.08
Agar	13.00

Add 78.08 g in 1000 ml of distilled water. Heat to boiling to completely dissolve the medium. Sterilize by autoclaving at 15lb (121°C) for 15 minutes.

### 4. SHEEP BLOOD AGAR

<u>Ingredients</u>	<u>g/litre</u>
Sheep blood agar base	40.50
Distilled water	1000 ml

Autoclave at 15lb (121°C) for 20 minutes. Cool the autoclaved base to 46-48°C and aseptically add 50ml of defibrinated sheep blood, mix properly and then pour into Petri plates.

## ABBREVIATIONS

WHO	World Health Organization
PALCAM	Polymixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol
BHIB	Brain Heart Infusion Broth
LSA	Listeria Selective Agar
OIE	Office International des Epizootis
AMM	Association of Medical Microbiologists
MDPH	Massachusetts Department of Public Health
NSS	Normal Saline Solution