ISOLATION, CHARACTERIZATION AND ANTIBIOTIC SENSITIVITY TEST OF PATHOGENIC *LISTERIA* SPECIES IN LIVESTOCK, POULTRY AND FARM ENVIRONMENT OF ODISHA*

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ABSTRACT

The present investigation was undertaken to study the occurrence of *Listeria* species in different animal and farms of Odisha. A total of 456 samples including 386 clinical samples and 70 environmental samples collected from different animals like cattle, sheep, goat, pig and poultry of different farms were screened for presence of *Listeria* species and 33 *Listeria* species were isolated which on pathogenicity testing revealed 5 *L. monocytogenes* and 3 *L. ivanovii* to be pathogenic. On biochemical characterization they were identified as *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* with prevalence rate of 1.75, 0.65, 1.75, 1.53, 1.31 and 0.21 percent respectively. Out of these isolates 5 *L. monocytogenes* and 2 *L. ivanovii* were found to be pathogenic in nature. Similarly prevalence of *Listeria* in different animals revealed 6.49% from cattle, 10% from sheep, 12.5% from goat, 15.83% from pig and 7.14% from poultry. Screening of environmental samples revealed isolation of 4 *Listeria* species with sewage having the highest prevalence followed by soil. Antibiotic sensitivity study indicated high susceptibility to ciprofloxacin, livofloxacin, amoxicillin, enrofloxacin where as high resistance were observed against gentamycin, penicillin G, amoxicillin, ceftriaxone, cephalexin and oxytetracycin.

Key words: *Listeria*, *L. monocytogenes*, *L. ivanovii*, Cattle, Domestic animals, Farm environment, Odisha.

INTRODUCTION

Listeriosis is an important emerging food borne zoonotic disease cause by pathogenic strains of *Listeria* species particularly *L. monocytogenes* and *L. ivanovii*. The infection has been reported in countries over six continents and the public health significance of the pathogen lies in its ubiquitous nature, wide host range, which includes 40 mammals, 20 birds, crustaceans, ticks and fishes and ability to persist for year in the environment (Unnerstad et al. 1996; Low and Donachie 1997). Among animals ruminants are most commonly affected and domestic animal species such as cattle, buffalo, sheep, goat, pig, poultry acts as intestinal carrier of listeriae, of which a significant proportion of animals sporadically shed the organisms in their feces or milk (Skovgaard and Morgen 1988). The pathogenic bacteria causes serious invasive disease that leads to abortion, mastitis, repeat breeding/infertility, encephalitis and septicaemia in animals and man (Malik, et al. 2002).

Reproductive disorders caused by infectious agents in animals are widely prevalent, and constitute an important problem in Indian situation. They are not only important from reproduction point of view but also potentially hazardous to human health because of zoonotic potential of important pathogenic agents frequently associated with such conditions. Systematic information on the role of listeric infection in causation of disease in animals is largely lacking in the Indian context, especially in Odisha. Moreover, many of the naturally occurring cases of listeriosis may go unnoticed or undetected due to lack of suitable diagnostic techniques employing specific media/antigen(s). So, it is in this context, the present investigation was envisaged to screen the clinical samples from animals for listeric infection by cultural methods. As environment is
regarded as the natural habitat for *L. monocytogenes* and acts as direct source of infection to animals and origin for contamination of foods so environmental samples were also screened for presence of *Listeria* species.

**MATERIALS AND METHODS**

**Collection of samples**

In the present study, a total of 456 samples including 386 clinical samples and 70 environmental samples collected from different animals like cattle, sheep, goat, pig and poultry of different farms were screened for presence of *Listeria* species. This includes 140 samples (70 vaginal swab and 70 faecal swab) from cattle of three organized dairy farms, 137 milk samples of cow, 30 faecal swabs of sheep, 24 faecal swabs of goats, 13 faecal swabs of pigs and 42 faecal swabs of poultry. To study prevalence of *Listeria* in animal farms 20 sewage, 20 soil, 15 water and 15 FYM samples were collected from three organized dairy farms and processed.

**Processing of samples**

Isolation of *Listeria* was attempted as per USDA method described by McClain and Lee (1988) after making necessary modification. Briefly, samples were enriched by two step enrichment procedure in University of Vermont medium (UVM) 1, followed by University of Vermont medium (UVM) 2 and then plating onto selective medium i.e. PALCAM agar.

**Enrichment of samples**

The swabs were directly transferred into sterile test tubes containing UVM-1 and incubated overnight at 37°C. 0.1 ml of the enriched inoculums was then transferred to UVM-2 and again incubated at 37°C for 48 hour.

**Plating on selective agar**

The enriched medium from UVM-2 broth was streaked directly on Polymyxin Acriflavin Lithium chloride Ceftazidime Esculin Mannitol agar (PALCAM). The petridishes we incubated at 37°C for 48 hours. The typical grey-green colonies with black centre and a black halo around the colonies were presumptively identified to be *Listeria* and subjected to further characterization and pathogenicity testing.

**Confirmation of the isolates**

The typical colonies were characterized by Gram’s staining, catalase test, oxidase test, tumbling motility at 20-25°C, MR- VP test, DLABN test and fermentation of sugar (rhamnose, xylose, maltose and mannitol). *In vitro* pathogenicity test was carried out by CAMP test with *Staphylococcus aureus* and *Rhodococcus equi*, haemolysis on 5% sheep blood agar and plating onto ALOA agar. The results were compared with *in vivo* pathogenicity test by mice inoculation test and chick embryo inoculation test.

**In vitro pathogenicity tests**

**Haemolysis on sheep blood agar (SBA) plate**

All the *Listeria* isolates were tested for the type and the degree of haemolysis on SBA. Briefly, the isolates were streaked onto SBA plates and incubated at 37°C in a humidified chamber for 24 h and examined for hemolytic zones around the colonies. The characteristic β-haemolysis in the form of wider and clear zone of haemolysis represented *Listeria ivanovii* while; a narrow zone of β-haemolysis was the characteristic of *Listeria monocytogenes*.

**Christie, Atkin, Munch-Petersen (CAMP) test**

All the *Listeria* isolates were tested by CAMP test as per the method of BIS (1994) with some modifications. Briefly, the standard strains of *Staphylococcus aureus* and *Rhodococcus equi* were grown overnight on sheep blood agar (SBA) plates at 37°C and their colonies were again streaked onto freshly prepared SBA plates having 7% sheep blood in a manner that these were wide apart and parallel to each other. Subsequently, the *Listeria* isolates were streaked onto these plates at 90° angle and 3 mm apart from *S. aureus* and *R. equi* strains before incubating them at 37°C for 24 h. The plates were examined for enhancement of haemolytic zone, if any, between a *Listeria* strain and the *S. aureus* or *R. equi* strain owing to the synergistic effect of their haemolysins in case of a CAMP-positive reaction. All the *Listeria* isolates with CAMP-positivity against *S. aureus* and *R. equi* were characterized as *L. monocytogenes* and *L. ivanovii*, respectively.

**In vivo pathogenicity tests**

**Mice inoculation test**

The pathogenicity testing of the *Listeria* isolates by mice inoculation test was performed according to the method described by Menudier *et al.* (1991) with suitable modifications. Briefly, the test isolates of *Listeria* were grown on BH slants at 37°C for 24 h. The bacterial growth was harvested
with sterile normal saline solution (NSS) and the opacity of inoculum was adjusted to McFarland Nephelometric tube number 1. Mice of either sex weighing 18-20 g were inoculated intraperitoneally with 0.4 ml of inoculum having approximately $10^7$ cfu of the test organism/ml. The inoculated mice were observed for mortality over a period of 72 h.

**Chick embryo inoculation test**

The pathogenicity of *Listeria* isolates was assessed by chick embryo inoculation test as per the method described by Notermans et al. (1991). Briefly, the blood vessel-free surface of chorioallantoic membrane (CAM) of two precandled 10-day old embryonated chicken eggs was inoculated with 0.1 ml of the test culture in BHI broth. The inoculated eggs along with the controls i.e. eggs inoculated with 0.1 ml of BHI broth were sealed with molten paraffin wax and incubated horizontally at 37°C for 3 days and were examined twice a day by transillumination for embryo death, if any. Any test isolate causing embryo mortality after 24 h of inoculation was considered as pathogenic.

**Antibiotic sensitivity test**

The antibiotic sensitivity tests were performed for each of the pathogenic *Listeria* isolates by employing the Bauer- Kirby diffusion method using antibiotic discs (HiMedia) as per the method of Bauer et al. (1966).

### RESULTS AND DISCUSSION

**Isolation of *Listeria* species from Cattle with History of Reproductive Disorder**

In present investigation, on microbiological analysis of a total of 140 samples from 70 cattle with history of reproductive disorder or mastitis, *Listeria* spp. was recovered from 9 samples. On biochemical characterization of these isolates pathogenic isolates of *L. ivanovii* were recovered from one (0. 71%) sample where as rest other 8 isolates were non-pathogenic. This includes *L. seeligeri* (3), *L. innocua* (2), *L. welshimeri* (2) and one *L. grayi* species. These results are comparable with that of Aghi et al. (2004) who reported isolation of one *L. ivanovii* and one *L. innocua* from (20) cattle faecal samples and Thakur (2000) isolated pathogenic *L. ivanovii* from abortion cases in U.P. Parihar et al. (2007) reported isolation of *Listeria* spp. from 20% of cervico-vaginal swabs which includes 10% of *L. monocytogenes* and 10% of *L. innocua* where as in the present study 4.28% of *Listeria* isolates were recovered (Table-1). This difference might be due to difference in location or in the method of collection of sample.

Screening of 137 milk samples, revealed isolation of 9 *Listeria* species, which were identified as four (2.91%) *L. monocytogenes*, three (2.18%) *L. innocua* and two (1.45%) *L. welshimeri*. On pathogenicity testing 2 of them were found to be pathogenic which has been reported. (Sarangi et al. 2009).

**Isolation of *Listeria* from reproductive disorder cases in sheep**

In sheep, only one pathogenic isolate of *L. ivanovii* was recovered from 30 samples in ewes and the rest two isolates were characterized as *L. seeligeri* (Table-2). *L. ivanovii* has been previously isolated from abortion cases in sheep from India (Chand and Sadana 1999). Although *L. monocytogenes* has been implicated as the most frequent cause of abortion in case of sheep (McLauchlin 1987), but in the present investigation no *L. monocytogenes* has been isolated.

**Isolation of *Listeria* from reproductive disorder cases in goat**

In the present study, 3 (12.5%) *Listeria* spp. were isolated from faecal swab of 24 does with history of reproductive disorders of which only one (4.16%) isolate was found to be characterized as *L. ivanovii* from biochemical tests and rest two are *L. welshimeri* but none of them were found to be pathogenic (Table-2). These observations are in agreement with Elezebeth et al. (2007) who reported isolation of

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Sample tested</th>
<th>No. of <em>Listeria</em> species isolated</th>
<th>Pathogenic <em>Listeria</em> isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal Swabs</td>
<td>179</td>
<td>17 (9.49%)</td>
<td>3 (1.67%)</td>
</tr>
<tr>
<td>Vaginal swab</td>
<td>70</td>
<td>3 (4.28%)</td>
<td>0</td>
</tr>
<tr>
<td>Milk</td>
<td>137</td>
<td>9 (6.56%)</td>
<td>2 (1.45%)</td>
</tr>
<tr>
<td>Sewage</td>
<td>20</td>
<td>3 (15%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Soil</td>
<td>20</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Farm Yard Manure</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Listeria spp. in 7.5, 5.56 and 14.52% of aborted, mastitic and apparently healthy goats respectively.

**Isolation of Listeria from pig**

In the present study, only two (15.3%) non-pathogenic *Listeria innocua* isolates could be recovered from faecal swab of 13 healthy pigs. These findings are comparable with Bonardi et al. (2002) who reported isolation of 14.7% of *L. innocua*, 1.3% of *L. grayi* and 0.7% of *L. seeligeri* from faeces of pigs. In our study no *L. monocytogenes* were isolated from faeces which were reported by Bonardi et al. (2002).

**Isolation of Listeria from poultry**

Listeriosis has been well reported in domestic fowls, where it causes septicemia and mortality. In India, Listeriosis in broiler chicken with history of mortality was reported by Vijay Krishna et al. (2000). Carrier status of *Listeria* species in indigenous birds in Kenya were studied by Njagi et al. (2004) where 2 *L. monocytogenes* and seven other *Listeria* spp. were recovered from oropharyngeal swab but none from cloacal swabs. However in our present study 3 (7.14%) *Listeria* spp. were isolated from cloacal swabs which were characterized as *L. monocytogenes*, *L. welshimeri* and *L. seeligeri*. On pathogenicity test, the *L. monocytogenes* isolate was found to be pathogenic.

**Isolation of Listeria from Environmental Samples**

Microbiological analysis of total 70 environmental samples comprising of sewage (20), soil (20), water (15) and FYM (15) of different farms were carried out for presence of *Listeria* species.

**Sewage**

High concentration of *L. monocytogenes* in sewage sludge may lead to contamination of crops on land and hence outbreak of human and animal listeriosis (Schlech et al. 1983). Of the sewage samples 3 (15%) *Listeria* isolates were recovered. On characterization two of them were *L. monocytogenes* and one found to be *L. innocua*. On pathogenicity testing both the *L. monocytogenes* isolates were found to be pathogenic. Giridhar and Garg (2002) isolated 16.6% of *Listeria* spp. from sewage samples which includes one each of *L. monocytogenes*, *L. ivanovii* and *L. welshimeri* which is in close agreement to this present finding. However Aghi et al. (2004) reported 7 (70%) of *Listeria* species from sewage of which four isolates were pathogenic *L. monocytogenes*. The reasons for such difference could be explained as varying environmental conditions between different locations.

**Soil**

Soil is regarded as the natural habitat for *L. monocytogenes* and acts as direct source of infection to animals and may act as origin for contamination of foods. Examination of the 20 soil samples showed presence of one *L. monocytogenes* which on pathogenicity testing was found to be non pathogenic in nature. Similar findings were also reported by Giridhar and Garg (2002) who found one *L. innocua* from 21 soil samples.

**Water**

*L. monocytogenes* has been reported in fresh water by many workers (Schaffter and Parriaux 2002; Nightangle et al. 2004) which may be due to contamination of water due to improper sewage disposal. However, no *Listeria* species were isolated from the 15 water samples examined in the present study, which may be due to small sample size.

**Farmyard manure**

*Listeria* species were not detected in any of the 15 samples of farmyard manure. The absence of *Listeria* might be due to destruction of micro-organism due to composting or desiccation of faecal matter (Aghi et al. 2004). However Al-Ghazali and Al-Azzawi (1990) reported that crops grown on soil treated with sewage sludge cake are contaminated with *L. monocytogenes*.

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>No. of sample tested</th>
<th>No. of <em>Listeria</em> species isolated</th>
<th>Pathogenic <em>Listeria</em> isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>277</td>
<td>18 (6.49%)</td>
<td>3 (1.08%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>30</td>
<td>3 (10%)</td>
<td>1 (3.33%)</td>
</tr>
<tr>
<td>Goat</td>
<td>24</td>
<td>3 (12.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Pig</td>
<td>13</td>
<td>2 (15.38%)</td>
<td>0</td>
</tr>
<tr>
<td>Poultry</td>
<td>42</td>
<td>3 (7.14%)</td>
<td>1 (2.38%)</td>
</tr>
<tr>
<td>Grand Total</td>
<td>386</td>
<td>29 (7.51%)</td>
<td>5 (1.29%)</td>
</tr>
</tbody>
</table>
The antibiogram of pathogenic isolate(s) of 5 *L. monocytogenes* reveals high sensitivity towards Ciprofloxacin and Livofloxacin which show 100% sensitivity. High sensitivity was also observed towards Amoxicilin and Enrofloxacin (80%) respectively, where as moderate sensitivity was observed towards Chloramphenicol and Amikacin (60%). The isolates show resistance towards Oxytetracyclin, Gentamycin and Cephadroxil (80% each), Penicillin G, Tobramycin, Cephotaxim and Cephalexin and Ceftriaxone (60% each). All the pathogenic isolates were resistant to Nalidixic acid. This result is in concurrence with the finding of Nigam et al. (1998) who reported that isolates of *Listeria* spp. from cow were cent per cent sensitive to ampicillin, chloramphenicol, penicillin and 88% sensitive to ciprofloxacin, gentamycin and 75% to tetracycline and all isolates to be resistant to nalidixic acid and furaxone. Similarly, antibiogram of two *L. ivanovii* isolates showed maximum sensitivity to most of the antibiotics except Tetracycline, ceftazidime, streptomycin, gentamicin, cephadroxil, nalidixic acid and penicillin-G. These results were in agreement with that of Kaur and Malik (2007), who reported that ciprofloxacin, gentamycin, cephalaxin and penicillin G to be highly sensitive to *L. ivanovii* isolates. According to the report of Phadke et al. (1979) 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. So, comparing the results with that of other workers shows that, in the last twenty years, a shift in the antibiotic sensitivity spectrum of *Listeria* has become evident and the alarming increase in resistance to conventional antibiotics like penicillin G, streptomycin, Gentamicin and Cephadroxil is posing a problem in the treatment of the disease.

**TABLE 3: Total prevalence of different *Listeria* species in Odisha.**

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Total No. of sample</th>
<th><em>Listeria</em> spp</th>
<th><em>L. monocytogenes</em></th>
<th><em>L. ivanovii</em></th>
<th><em>L. seeligeri</em></th>
<th><em>L. welshimeri</em></th>
<th><em>L. innocua</em></th>
<th><em>L. grayi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>277</td>
<td>18</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Sheep</td>
<td>30</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Goat</td>
<td>24</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pig</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Poultry</td>
<td>42</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Environment</td>
<td>70</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Grand Total</td>
<td>456</td>
<td>33 (7.23%)</td>
<td>8 (1.75%)</td>
<td>3 (0.65%)</td>
<td>6 (1.31%)</td>
<td>7 (1.53%)</td>
<td>8 (1.75%)</td>
<td>1 (0.21%)</td>
</tr>
</tbody>
</table>

**CONCLUSION**

In the present study, a total of 33 *Listeria* species isolates were recovered from 456 samples processed, so the overall prevalence rate of *Listeria* species turned out to be 7.23%. On biochemical characterization they were identified as *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* (Table- 3). Out of these isolates 5 *L. monocytogenes* and 2 *L. ivanovii* were found to be pathogenic in nature. Similarly prevalence of *Listeria* in different animals reveals 6.49% from cattle, 10% from sheep, 12.5% from goat, 15.83% from pig and 7.14% from poultry (Table-2). Screening of environmental samples revealed isolation of 4 *Listeria* species with sewage having the highest prevalence followed by soil. Antibiogram study indicated that in the treatment of Listeric infection ciprofloxacin, livofloxacin along with amoxicillin and enrofloxacin can be used as primary choice of therapy but not but Gentamicin, penicillin G, Cephadroxil, Ceftriaxone, Cephotaxim, Oxytetracycin which are commonly used in field conditions.

Hence the study highlights the prevalence of *Listeria* species in different animals of Odisha, which may be the cause of reproductive diseases seen in animals. So, a more detailed study regarding the role of *Listeria* in reproductive disorder cases should be carried out and as isolation of *Listeria* is a tedious and time consuming job, so quick and reliable detection methods using molecular biology should be used in future. Considering the zoonotic potential of the pathogen, there is an urgent need of hygienic measures to be adopted at farms and public awareness must be raised regarding the proper disposal of the sewage, use of pasteurized milk and avoiding the entry of *Listeria* in food chain.
REFERENCES


