Isolation of *Listeria monocytogenes* and anti-listeriolysin O detection in sheep and goats

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Abstract

Studies were undertaken to compare the detection of anti-listeriolysin O antibodies (ALLO) and isolation of *Listeria monocytogenes* from meat and milk samples of sheep and goats. Out of 201 samples (87 of milk and 114 of meat) tested, 17.64% yielded *Listeria* species isolates. *L. monocytogenes* was isolated from 6.66 and 1.56% of meat (60) and (64) milk samples of goats, respectively, and 7.4% of sheep meat (54) samples. All the samples of ewes’ milk (23) were negative for *L. monocytogenes*. Seropositivity for ALLO was observed in 41.13 and 33.76% of goats and sheep, respectively. The culture positivity for *L. monocytogenes* and detection of ALLO did not show any agreement ($\kappa<0.202$).

Keywords: Detection; *Listeria monocytogenes*; Listeriolysin O; Goats; Sheep

1. Introduction

Listeriosis is an important bacterial zoonosis caused by an intracellular pathogen — *Listeria monocytogenes*. Listeric infections occur in a variety of animals including man. Cases of listeriosis arise mainly from the ingestion of contaminated food and the disease is particularly common in ruminants fed on silage (Low and Donachie, 1997). Listeriosis may occur in several forms, including encephalitis, abortion and septicaemia. *L. monocytogenes*, has been implicated in several foodborne outbreaks and has been isolated from dairy products, meat, poultry, fish, vegetables and food processing plants (Gelline and Broome, 1989).

Listeriolysin O (LLO), an extracellular 58 kDa haemolysin, is a major virulence factor of *L. monocytogenes* (Gaillard et al., 1986) and is produced by all the pathogenic strains (Geoffroy et al., 1989). LLO has been identified as a candidate antigen for a serological assay (Low and Donachie, 1991), and could be a dominant antigen target of anti-listerial immunity (Bouwer et al., 1992). Antibodies to LLO (ALLO) were shown to be reliable indicators for serodiagnosis of listeric infections in sheep by immunoblotting (Low and Donachie, 1991) and by ELISA (Low et al., 1992). This paper reports the use of LLO based ELISA to detect *L. monocytogenes* infection in apparently healthy sheep and goats.
2. Materials and methods

2.1. Bacteria

*Listeria monocytogenes* MTCC 1143 (NCTC 11994) was obtained from the Institute of Microbial Technology, Chandigarh, India. The organism was maintained on tryptose phosphate agar slopes at 4°C and used for preparation of LLO.

2.2. Samples

A total of 402 samples comprising 201 serum samples (124 from goats and 77 from sheep) and corresponding 201 samples either of meat (from 60 goats and 54 sheep) or milk (from 64 goats and 23 sheep) from the same animal case were collected randomly at slaughter houses of Delhi and Bareilly (India). The meat samples were collected mainly from male animals.

The serum samples were collected in sterile vials, transported on ice from the place of their collection and stored at −20°C until tested. The milk/meat samples were collected in sterile test tubes/UV sterilised polyethylene sachets, transported on ice and stored at 4°C till processed for microbiological analysis.

2.3. Enrichment and isolation of Listeria

Isolation of *Listeria* was attempted from the collected meat/milk samples as per the USDA method described by McClain and Lee (1988) after some modifications.

Approximately 5–10 ml of each of milk sample was directly inoculated into 50 ml University of Vermont Medium (UVM1), and incubated overnight at 30°C. Meat samples (≈10 gm each) were instead placed in a sterile polyethylene sachet containing 90 ml UVM1 and mixed thoroughly in Stomacher (Model BA 6021, Seward Laboratory, London) for 5 min and incubated overnight at 30°C. Then, 0.1 ml of the enriched inoculum from UVM1 was transferred to UVM2 and again incubated overnight at 30°C. The enriched inoculum from UVM2 was streaked onto Dominguez–Rodriguez isolation agar (DRIA) and plates were incubated at 30°C for 48 h.

The greenish-yellow, glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis were considered to be of *Listeria*. The presumed colonies of *Listeria* (at least three per plate) were further confirmed.

2.4. Confirmation of the isolates

Morphologically, typical colonies were verified by Gram’s staining, catalase reaction, tumbling motility at 20–25°C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *Staphylococcus aureus*, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and α-methyle-D-mannopyranoside) and haemolysis on 5% sheep blood agar.

The isolates found as rhamnose and MR-VP positive, and xylose and nitrate negative were tested for their pathogenicity by mice inoculation.

2.5. Mice inoculation test

Mice inoculation test was performed according to the method described by Menedier et al. (1991). Mice of either sex weighing 18–20 gm were inoculated intraperitoneally with 0.4 ml of inoculum having ≈10⁷ CFU of the test organism/ml. The mice were observed for mortality over a period of 72 h.

2.6. Isolation and purification of LLO

LLO was prepared and purified from the cell free supernatant by ion-exchange chromatography in accordance with the method of Lhopital et al. (1993). The purity of the LLO was checked by SDS-PAGE, which showed it to be a homogeneous 58.0 kDa protein. The fractions having LLO were pooled and the protein content was estimated, and finally stored at −20°C until used.

2.7. ELISA

The indirect-ELISA was performed as per the method of Low et al. (1992). The ELISA was standardised by checker board analysis. Briefly, purified LLO was used in at the optimal concentration of 40 ng per well (100 μl per well) for coating the microtitre plates (Nunclon, Denmark). The plates were covered and incubated at 37°C for 2 h before washing five times with phosphate buffer saline (PBS), pH 7.2 plus
0.05% Tween 80 (PBS-T). Each of the test serum was diluted 1:200 in PBS and added (100 µl per well) to the plates. The sealed plates were incubated at 37°C for 90 min and again washed as before. Subsequently, horseradish peroxidase conjugates (HRPO) diluted in PBS were added (100 µl per well) to the plates. The conjugates were diluted as, rabbit anti-goat HRPO (1:6000; National Institute of Immunology, New Delhi) and rabbit anti-sheep HRPO (1:2000). The plates were again incubated at 37°C for 90 min and washed as described earlier. Finally, o-phenylenediamine dihydrochloride (OPD) solution (1 mg/ml) in citrate buffer with 12 µl/100 ml of hydrogen peroxide was added (100 µl per well) as a substrate and after 15 min, the reaction was read by ELISA plate reader (Anthos Labtek) at 492 nm. Each sample was tested in duplicate. Samples showing a positive to negative optical density ratio of 2.0 or above in serum dilution of 1:200 were considered as positive.

2.8. Analysis of data

The data was analysed for test agreement (Martin et al., 1993).

3. Results

3.1. Isolation of listeriae

Listeria sp. were isolated from 21.66% of 60 meat samples and 9.37% of 64 milk samples from goats. Out of these four (6.66%) and one (1.56%) isolates were confirmed as L. monocytogenes from meat and milk, respectively (Table 1).

Out of 54 meat and 23 milk samples from sheep, 18.51 and 30.43% were positive for listeriae, respectively. Four (7.4%) isolates from meat samples were confirmed as L. monocytogenes. All the samples from ewes’ milk turned out to be negative for L. monocytogenes (Table 1). From milk and meat samples in all, nine isolates were confirmed as L. monocytogenes.

3.2. Pathogenicity testing

All the nine L. monocytogenes isolates were pathogenic to mice. The liver and spleen of dead mice showed considerable congestion and the pathogen was re-isolated from the liver and spleen of dead mice.

3.3. Detection of anti-listeriolysin O (ALLO) antibodies

ALLO antibodies were detected in the sera of sheep and goats tested by indirect-ELISA (Table 2). Anti-LLO titres were higher in case of goats (absorbance$_{492}$ >1.212).

3.4. Isolation rate and anti-listeriolysin O (ALLO) positivity

LLO based indirect-ELISA did not agree ($\kappa$=0.0–0.202) with the cultural isolation of L. monocytogenes in cases of goats and sheep. All the five L. monocytogenes positive goats had high ALLO titres. Of the 14 cases of goats showing cultural positivity for Listeria sp. other than L. monocytogenes, seven (50%) demonstrated seropositivity for ALLO. However, the remaining seven (50%) culture positive goats were found negative for ALLO. Out of 105 goats with cultural negativity, 39 (37.14%) were found as positive for ALLO, whereas 66 (53.22%) were negative.

Of the four sheep culturally positive for L. monocytogenes, only two demonstrated the presence of

<table>
<thead>
<tr>
<th>Animals</th>
<th>Milk</th>
<th>Meat</th>
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<tr>
<td></td>
<td>Sample no.</td>
<td>Listeria sp. (%)</td>
</tr>
<tr>
<td>Goat</td>
<td>64</td>
<td>7.81</td>
</tr>
<tr>
<td>Sheep</td>
<td>23</td>
<td>30.43</td>
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<tr>
<td>Overall</td>
<td>87</td>
<td>13.79</td>
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ALLO. Out of 13 cases showing cultural positivity for *Listeria* sp. other than *L. monocytogenes*, five (38.5%) demonstrated seropositivity for ALLO. However, eight (61.5%) culture positive sheep were found negative for ALLO. Out of 60 sheep with cultural negativity, 19 (31.66%) were found as positive for ALLO, whereas 41 (53.24%) were negative.

### 4. Discussion

The percentage culture positivity of *L. monocytogenes* in milk observed in the present study is in agreement with the reported incidences in milk as 2.2% (Farber and Peterkin, 1991), 3.48% (Ryser and Marth, 1991) and 5.78% (Bhilegaonkar et al., 1997). The observed positivity for the pathogen in goats’ milk agrees with the reported rate of 0.8% from UK (Greenwood et al., 1991) and 7.8% from Yugoslavia (Katic and Stojanovic, 1992).

In the present study, in spite of high isolation rate of *Listeria* sp. from ewes’ milk (30.43%), *L. monocytogenes* was not detected. This is consistent with the findings that most of the sheep farms (93.38%) in Spain produced milk free from *L. monocytogenes* throughout 1 year sampling period (Rodriguez et al., 1994). In the Indian context, sheep are often reared by extensive grazing where the chances of their exposure to dampness favouring growth of the pathogen are minimised.

The isolation rates of *Listeria* sp. and *L. monocytogenes* in meat were similar to reports where the recovery rates of *L. monocytogenes* have been reported as 0–80% from raw meat and 7–80% from European ground meat (Ryser and Marth, 1991), and 0–100% from fresh meat samples in USA (Buchanan et al., 1989). The large variations in the reported incidence rates may be partly due to differences in methods of detection including the factors like procedures used, the sample size, the number of single colony isolates taken to confirm the presence of haemolytic colonies and the source of samples (Farber and Peterkin, 1991).

In the present study, a high percentage of positivity for ALLO was detected by indirect-ELISA in goats and sheep. These results can be explained in the light of published reports indicating seroconversion to ALLO after oral or subcutaneous infection in sheep (Low and Donachie, 1991; Lhopital et al., 1993). The high positivity for ALLO in the absence of any clinical disease observed in our study may possibly be attributed to exposure of these animals to low infective doses which has been reported to elicit persistent immune response to LLO equal to that with higher infective doses (Lhopital et al., 1993) through various established sources of infection including poor quality silage (Low et al., 1992) and vegetables (deSimon et al., 1992; Arumugaswamy et al., 1994).

The results of the present study are in accordance with the findings reporting the detection of ALLO in naturally infected sheep with a history of enteritis and abortions (Low et al., 1992). In the present study, ALLO could not be detected in two cases which were culture positive for *L. monocytogenes*. The reason for this discrepancy was not apparent. ALLO was detected, however, in serum samples of animals culturally negative for *L. monocytogenes*. Studies on kinetics of ALLO production following experimental infection with pathogenic *L. monocytogenes* of sheep (Lhopital et al., 1993), goats (Miettinnen et al., 1990; Banu Rekha, 1997) and buffaloes (Choudhari, 1997) showed that faecal, nasal and blood cultures became negative for the pathogen as ALLO peaked in the sera of infected animals. In these studies, despite the failure of the recovery of *L. monocytogenes* from cultures, significant levels of ALLO titres were detected up to long periods.

Listeriolysin-O (LLO) has been known to be a major virulence factor produced by all pathogenic strains of *L. monocytogenes* (Low et al., 1992) and is known to induce T-cell recognition during the course of an acute listeric infection (Berche et al., 1987). Also specific antigenic epitopes in LLO have been reported (Nato et al., 1991). Culturing of all the samples for the pathogen is costly and cumbersome. Therefore, LLO as an antigen can be employed in serological assays for epidemiological surveys.
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References


