Short Paper

Isolation and identification of *Pasteurella hemolytica* biotype A from sheep in Urmia, Iran

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Summary

The present study is the result of a survey carried out between May 1, 1998 and April 30, 1999, examining a total of 1,988 lung tissue samples bacteriologically and pathologically for the presence of ovine pasteurellosis in Urmia, Iran.

*Pasteurella hemolytica* biotype A was isolated from 316 (16%) lung tissues. The isolates were confirmed on the basis of morphological and biochemical criteria. Biotyping of *P. hemolytica* strains was based on the cultural, biochemical and pathogenic characteristics.

The description of gross lesions appeared to be an obvious severe fibrinous pneumonia in anteroventral part of the lung. Acute fibrinous pneumonia was a common finding and considered as the most impressive lesion. The amount of fibrinous exudate deposited on the pleural surface was found to be heavy or variable. This is the first recorded isolation of *P. hemolytica* biotype A from sheep in Urmia, Iran.

Significant relationship was documented between isolation rate and season of the year. Ovine pasteurellosis had a seasonal variation with higher rates in Winter (41.2%) and Spring (29.0%) than those in Summer (20.8%) and Fall (12.0%).

Key words: Sheep, Lung, Fibrinous pneumonia, *Pasteurella hemolytica*; biotype A

Introduction

Pasteurellosis is one of the oldest diseases affecting domestic animals. Nonetheless, it still remains a major problem for over sixty years, among veterinarians and farmers (Marsh, 1953; Midgley, 1966; Biberstein and Thompson, 1966; Carter, 1968; Tizard, 1992).

In farm animals, ovine pasteurellosis is observed in all major sheep-producing areas including southern and northern Africa, Argentina, Peru, Chile, United Kingdom, the Middle East, Iran, Mongolia, New Zealand and North America (Frederiksen, 1973; Gilmour and Gilmour, 1989; Woolcock, 1992).

Like many potential pathogenic microorganisms, *Pasteurella hemolytica* is often detected in the upper respiratory tract and the upper part of the digestive tract of apparently healthy animals and does not seem to cause any disease until the presence of a predisposing factor such as driving or shipping to feedlot, winter pastures and climatic changes (Jones, 1921; Carter, 1957; Biberstein and kennedy, 1959; Jones, 1960; Timoney *et al*., 1988; Tizard, 1992). Biotype A of *P. hemolytica* causes pneumonia in all ages of sheep and septicemia in young lambs. Biotype T, on the other hand, is associated with a distinct septicemia syndrome in young adult sheep (De Alwis, 1999).

This study presents the status of ovine pasteurellosis in Urmia, northwestern Iran.
Materials and Methods

Source of Isolates
There were reports of clinically-evident respiratory disease of sheep in Urmia, Iran. Examination of the lungs of slaughtered sheep aged between 1 to 7 years, was carried out at an abattoir between May 1, 1998 and April 30, 1999. Lungs with gross lesions in favor of pneumonia were collected.

Those sheep died of illness underwent necropsy at the Veterinary Clinic of Urmia University. At necropsy, specimens with pneumonic lung lesions and pleuritic exudate were collected. Duplicate specimens were taken for bacteriologic and pathologic examinations.

Bacteriology
Cultivation and isolation of Pasteurella spp.
A portion of the specimens taken from the apical, cardiac and diaphragmatic lobes of the affected lungs were collected. These samples were placed into a plastic bag and transported in containers packed in ice.

The samples were taken to the Microbiology Laboratory, College of Veterinary Medicine, Urmia University. A section measuring one or two inches, of the apical, cardiac and diaphragmatic lobes of the lungs were aseptically removed, immersed in alcohol, opened and minced with sterile forceps and inoculated in 10 ml trypticase soy broth (TSB). The TSB was then incubated for 2 hours at 37°C and then streaked into blood agar plate (BAP; 5% blood in blood agar base). The BAP was incubated at 37°C for 24 hours. Based on the colony characteristics and appearance, the suspected colonies were then isolated. Selected colonies were transferred to brain heart infusion (BHI) using a sterile toothpick. After 24 hours, BHI culture media were observed for any growth. The positive media were then subcultured on BAP and incubated at 37°C for another 24 hours.

Antibiotic Sensitivity Test
Antibiotic sensitivity test was performed on Mueller-Hinton agar using the Kirby-Bauer Method (Bauer et al., 1966). Commercially available antibiotic impregnated discs were used. At least 3–5 pure colonies of the isolated bacteria were transferred to 5–10 ml of sterile physiological saline solution using a vortex mixer or by shaking. Using a sterile glass spreader or swab, a drop of the suspension (0.2 ml.) was then spread evenly on Mueller-Hinton agar plates. The plates were inverted and incubated at 37°C for 18–24 hours. Each plate was examined and the zones of complete inhibition were measured using a caliper or ruler. The results were compared with a standard antibiotic discs chart.

Table 1: Seasonal variation in infection with P. hemolytica biotype A as isolated from sheep in Urmia, Iran

<table>
<thead>
<tr>
<th>Season</th>
<th>Total no. of samples (+) for P. hemolytica</th>
<th>Total no. of lung samples</th>
<th>Mean Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td>184</td>
<td>634</td>
<td>29.02% a</td>
</tr>
<tr>
<td>Winter</td>
<td>174</td>
<td>422</td>
<td>41.23% b</td>
</tr>
<tr>
<td>Summer</td>
<td>121</td>
<td>582</td>
<td>20.79% c</td>
</tr>
<tr>
<td>Fall</td>
<td>42</td>
<td>350</td>
<td>12.00% d</td>
</tr>
</tbody>
</table>

a, b, c, d; Means with different superscripts differ significantly (P<0.05).
Pathology
The apical, cardiac and diaphragmatic lobes of the lungs were examined. Based on the color, consistency and appearance on cut surface of the samples, those specimens with red hemorrhagic foci, consolidation and those with frothy mucoid or purulent exudate oozing out from bronchi, bronchioles or cut surfaces were selected. The gross pathological features were recorded. The selected specimens were then fixed in 10% buffered formalin, dehydrated and embedded in paraffin, sectioned using a microtome at 6 millimicron, stained with hematoxylin and eosin and examined under light microscope to diagnose the nature of the lesions.

Results
Sample Received
A total of 1,988 lung samples were received from different towns and villages of Urmia, Iran, between May 1, 1998 and April 30, 1999. One thousand and four hundred forty (1,440) samples were collected from the government owned abattoir and 548 samples from clinical cases. Sheep aged between 1 and 7 years.

Bacteriology
Out of 1,988 lung tissue samples examined, only 316 (16%) isolates were identified as *P. hemolytica* biotype A. These were diagnosed based on the morphological characteristics, i.e., pinpoint colonies, less mucoid, coalescent and a zone of beta hemolysis, at least 1 mm in diameter surrounding the colonies on the blood agar (Montgomery *et al*., 1938; McGowan *et al*., 1957). On McConkey agar plate, there were some pink round and smooth colonies 0.8–1 mm in diameter. Microscopically, the organisms were all bipolar Gram negative rods (Shreeve *et al*., 1970).

Biochemically, possible *Pasteurella* colonies were streaked and stabbed on triple sugar iron (TSI) agar. The TSI slants then were observed for the typical reaction caused by *Pasteurella* spp. Five hundred samples were further run for indole, MR-VP and citrate utilization. Only 340 samples reacted as indole (-), MR (+), VP (-) and citrate utilization (+).

Arabinose, lactose, raffinose, salicin, maltose, trehalose and xylose fermentation tests were positive for all 316 isolates without gas formation (Timoney and Gillespie, 1981).

Antibiotic Sensitivity of the Isolates
Susceptibility to penicillin G was used as criterion for biotyping. In this study, 320 of the 400 isolates of biotype A were sensitive to penicillin. Penicillin sensitivity of this isolates may have resulted from the frequent use of the antibiotic against ovine pasteurellosis in the country (McGowan *et al*., 1957; Smith, 1959; Bauer *et al*., 1966; Dikova, 1976).

Pathology
Gross Lesions
A noteworthy feature of this study was the presence of severe fibrinous pneumonia in anteroventral third of the lungs. A detailed description of a gross section of the pneumatic lobe exhibits advanced and fatal lesions involving most ventral tissue of the cranial (apical), intermediate (cardiac) and caudal (diaphragmatic) lobes. The lungs were characterized by well-demarcated dark areas of consolidation covered by a layer of fibrin. Upon incision, the dark areas showed well-circumscribed hemorrhagic areas with some creamy red tinge exudates coming out from the bronchi and bronchioles. Mucopurulent exudates oozed out from the bronchioles and bronchi. Pleuritis was also evident grossly.

Histopathology
Histopathological examination of the lungs with pneumonic pasteurellosis showed acute fibrinous pneumonia with pleuritis. The lesions were characterized by irregular-shaped areas of neutrophil and lymphocyte infiltration with only a few macrophages. The bronchi and bronchioles were desquamated and were plugged by inflammatory exudates and cellular debris. They were also surrounded by inflammatory cells. The presence of fibrin strands in the alveoli and pathognomonic oat cell reaction were striking.

Statistical analyses using SPSS and Duncan’s multiple range test (n=1, 988; F=34.0042; P<0.0005) showed that there is a
significant seasonal variation in infection with *P. hemolytica* biotype A (Table 1). The highest percentage of *P. hemolytica* was observed during winter (41%–23%) while the lowest was in fall (12%).

**Discussion**

Problem may arise in isolating *P. hemolytica*, since this organism is easily over grown by other organisms. Because of its typical swarming characteristics on BAP, the earliest possible contamination seen on the plate is that caused by *Proteus mirabilis*. Factors producing contamination include improper handling and preparation of the samples, use of inappropriate sterilized cotton swabs and finally, the presence of normal bacterial flora in different parts of the respiratory tract.

Based on the cultural growth of the isolates on BAP and their biochemical characteristics, the isolate was shown to be *P. hemolytica*. Careful observation of the texture of the colonial surface one of the most diagnostic features revealed a smooth, less mucoid, grayish pinpoint dewdrops-shaped colony. These colonial properties are reflection of certain bacterial characteristics and are attributed mainly to the presence of capsules. Capsular materials promote cellular orientation, hence, the smoothness and mucoid appearance of the growth on BAP (Stanier et al., 1965). *P. hemolytica* can grow on McConkey agar plates and is able to ferment lactose.

Typically, coccobacillary and bacillary *P. hemolytica* have darkly stained ends giving it a bipolar appearance, a phenomenon that was evident in the Gram stained isolates of this study.

In our study, the recovery rate of *P. hemolytica* biotype A was only 16% as compared to the very high rates of pure isolations reported from abroad. This very low recovery rate of the organism, even in severe cases of pasteurellosis observed in the laboratory and in the slaughterhouse could be attributed to a number of factors including 1) antibiotic therapy, 2) combination with other related bacteria such as *Proteus spp.* and *Bacillus spp.* which masked off the presence of the organisms. 3) and chronicity of the lesions that promoted growth of other bacteria. The focus of the present study in this country was to determine the real extent of *P. hemolytica* biotype A infection in sheep. Results of this study will help in formulating better control measures against ovine pasteurellosis. Perhaps, the inclusion of biotype A is necessary in the present vaccine.

**Acknowledgment**

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**References**