Isolation, Characterization and Antibiogram of *Mycoplasma bovis* in Sheep Pneumonia

1Amit Kumar, 2Amit K. Verma, 3Neeraj K. Gangwar and 4Anu Rahal
1Department of Veterinary Microbiology and Immunology, 2Department of Veterinary Epidemiology and Preventive Medicine, 3Department of Veterinary Pathology, 4Department of Veterinary Pharmacology and Toxicology, College of Veterinary Sciences and Animal Husbandry, Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishvidyalaya Wvum Go anusandhan Sansthan (DUVASU), Mathura-281001, India

Corresponding Author: Amit K. Verma, Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Sciences and Animal Husbandry, Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishvidyalaya Wvum Go anusandhan Sansthan (DUVASU), Mathura-281001, India

ABSTRACT

*M. bovis* is a common respiratory pathogen for cattle but it may produce similar disease complex conditions. It can be transmitted through litter, tools and the hands and clothing of the owners and handlers in sheep and goats. In comparisons to other mycoplasmas *M. bovis* shows relatively high resistance under some environmental conditions. The occurrence in sheep is rare but it causes severe economic losses. The present study revealed an outbreak of pneumonia in sheep flock leading to high morbidity and mortality. To get an idea of etiological agent of outbreak the samples were collected from morbid animals and during the postmortem from dead animals. Nasal and tracheal swabs, blood, serum, faecal samples and tissues from multiple organs were collected and examined in laboratory. The *M. bovis* was the etiological agent isolated from nasal swabs and lungs of infected and dead animals, respectively. Histopathological findings also supported mycoplasmic lesions in dead animals. As the success of treatment of the respiratory diseases due to *M. bovis* depends on the right choice of the compound, its distribution in the tissues and last but not least the simultaneous antibacterial effect in secondary and mixed infections, the *in vitro* antimicrobial drug sensitivity was performed to select the drug of choice for treatment. The antibiogram of isolate revealed Tylosin and Enrofloxacin with remarkable zone of inhibition. Therefore, tylosin was recommended as drug for treatment and flock recovered with in the period of 15 days. There was no further mortality and all the infected sheep recovered from the signs of illness. This seems to be the first report of *M. bovis* outbreak in sheep in country.

Key words: *Mycoplasma bovis*, enrofloxacin, pneumonia, sheep, tylosin

INTRODUCTION

Sheep pneumonia is regarded as a complex disease, involving interaction among host, (immunological and physiological), multiple agents, (bacterial, viral, mycoplasma) and environmental factors (Brogden et al., 1998). These can affect any age group of animal but most commonly occurs in lambs aged between 3-12 months when acquired maternal antibody titres are reduced. Cases of chronic pneumonia usually present with ill thrift and varying degrees of
respiratory compromise, with respiratory distress usually evident during exercise (Bell, 2008). Respiratory problems are one of the most common and relevant diseases of sheep in all the sheep-rearing countries. They cause stunted growth, mortality in lambs and important economic impact due to drug costs and condemnations of meat in abattoirs (Jones et al., 1982; Goodwin et al., 2004). In many countries they play important role in socioeconomics (Ajala et al., 2008). Out of many etiological agents Mycoplasma species, particularly M. bovis, is harbored in the respiratory tract without showing any apparent clinical symptoms by clinically healthy young cattle (Kumar et al., 2011) and shed through their nasal discharge for months or years (Ter Laak et al., 1992). M. bovis can also colonize sheep (Bocklisch et al., 1987) and goat (Egwu et al., 2001) and the pathogen can be transmitted among these animals. M. bovis shows relative high resistance under some environmental conditions (Nagatomo et al., 2001). The success of treatment of M. bovis in sheep mainly depends on the choice of the compound used for the treatment, its distribution in the tissues and last but not least the simultaneous antibacterial effect in secondary and mixed infections of e.g. Pasteurella multocida, Mannheimia haemolytica or Haemophilus somnus (Shayegh et al., 2008; Poumarat et al., 1994). In this context, present study revealed the involvement of M. bovis in sheep pneumonia and the selection of antibiotics for timely recovery of infected animals to prevent economic losses due to mortality and morbidity in sheep flocks.

MATERIALS AND METHODS

Study area, animal and management: The incidence occurred in two flocks of sheep near city Chatta block in district Mathura, located in Uttar Pradesh, India. The sheep population of flocks was 270 at the time of incident. The age of most of the affected animals was between 6 months to two years. The animals were grazed during the day time on natural pasture and had free access to hay (made of natural pasture) at night in properly sheltered pens. Those animals that were kept together during the day time were mostly sheltered at night in same pen. Animals suffering from respiratory disease were supplemented with concentrate feed in separate pen. They had free access to water. All the sheep were drenched against internal parasites with the combination of albendazole and ivermectin approximately one month before the onset of outbreak. Animals were not vaccinated against any disease.

History, clinical examination and data collection: An outbreak with respiratory and nervous signs occurred in the month of August 2010. Infected and suspected animals were thoroughly examined by animal health researchers of Veterinary University, Mathura for pyrexia (rectal temperature more than 105°F), abnormal respiration (sternal and abdominal respiration, polypnoea, dyspnoea), coughing and nasal discharge. Other signs like anorexia, staggering and paresis before death were also recorded. The health and basic epidemiological information viz., morbidity, mortality and case fatality rate were collected and analyzed for occurrence of the disease. Clinical, serological, gross pathological and bacteriological investigations were carried out from infected and post-mortem and histopathological examinations were conducted from dead animals.

Sample collection: Nasal and tracheal swabs, feces, blood and serum samples, were collected from sick animals whereas tissues from spleen, liver, lung, kidney, lymph nodes and hearts were taken from dead animals during post-mortem.
Postmortem and Histopathological examination: Post-mortem examination was conducted immediately after death. During autopsy, special emphasis was given to respiratory organs. Three animals, found dead were subjected to thorough post mortem examination. Necropsy was conducted by the method described by Thompson (1978). Portion of the internal organs of dead lambs (mostly lungs, also spleen, liver, kidney, lymph nodes and heart) were collected for microbiological and histopathological examination aseptically. Standard aseptic procedures were followed for microbial isolation; the surface of the organs were cleaned with denatured alcohol and flamed immediately following the procedures of Carter et al. (1995) followed by searing with heated spatula before the inner tissues were chopped and collected in PBS (pH 7.4) and further inoculated into sterile screw capped test tubes containing 5 mL of Tryptose soya/PPLO/SDA broth.

Microbiological culture and identification: Microbiological isolation was performed from nasal swabs and blood samples of sick sheep and internal organs of dead sheep (mostly lungs, also spleen, heart, liver, kidney and lymph nodes). Samples and swabs were inoculated onto nutrient agar, MacConkey Lactose Agar (MLA), Sabouraud’s Dextrose Agar (SDA) and 5% sheep blood agar plates and incubated aerobically/microaerobically at 37°C for 24 h (Quinn et al., 2002). After 24 h incubation, the plates were observed for presence of bacterial and fungal colony. Swabs for mycoplasma isolation were homogenized in 1 mL PBS then 100 μL of this solution was transferred into PPLO broth enriched with horse serum under 37°C, 5% CO₂ for 48 h. (Rosengarten et al., 1994) afterwards. Samples from PBS (pH 7.4) were triturated, filter sterilized (0.22 micron syringe filter) and inoculated on MDBK cell lines at 36°C, 5% CO₂ for 48 h in CO₂ incubator.

Lung samples for the isolation of mycoplasma were cultured according to the method described by Ter Laak et al. (1992). For mycoplasma slight color change of broth media due to pH shift was examined daily. The cultures were inoculated onto solid Medium using the running drop technique on days 2 and 5 (Lauerman, 1994). The agar plates were incubated at 37°C in 5% CO₂ atmosphere for 14 days. The plate were examined under stereomicroscope and presumptive identification of isolated mycoplasma was made by traditional physiological and biochemical methods (Erno and Stipkovits, 1973a, b).

Serological confirmation of mycoplasma culture: The species-specific identification was performed with anti-M. bovis hyperimmune serum which was used in growth inhibition and metabolic inhibition tests (Lauerman, 1994). Identification of M. bovis was also performed by DID and slide agglutination test (Bradbury, 1998). Mycoplasma antigen was prepared as per the method described by Kumar (2000).

Antibiogram: Mycoplasma colonies isolated from lung tissues were tested for antimicrobial susceptibility testing by the Bauer et al. (1966) following the NCCLS (2002) guidelines. The following commonly used antimicrobial agents (Hi-Media, Mumbai) were used at the indicated concentrations (μg/disc except where specified) viz., Amikacin (30 g), Amoxyceillin-clavulanic acid (20/10 μg), Ampicloxacillin (10 μg), Ciprofloxacine (30 μg), Chloramphenicol (30 μg), Enrofloxacin (10 μg), Erythromycin (15 μg), Levofloxacine (5 μg), Streptomycin (10 μg), Tetracycline (30 μg), Penicillin (10 IU), Amoxicillin (30 μg), Cefotaxime (30 μg), Ceftriaxone (30 μg), Tiamulin (20 μg), Tylosin (20 μg) and Kanamycin (30 μg).

RESULTS AND DISCUSSION
Microbiological examination of nasal swab, blood and tissues of different organs for infectious agent revealed no growth in blood agar, MLA, MDBK cell lines and SDA as there was no bacterial
Fig. 1: Edema and haemorrhages

Fig. 2: Exudation and edema in bronchi

growth, cytopathic effects and fungal colonies, respectively. These were suggestive of no involvement of bacteria, virus and fungi. However, PPLO broth revealed change in color without turbidity after the 48 h of incubation. It was suggestive of mycoplasma growth so it was further incubated. After 72 h of incubation PPLO broth was streaked on PPLO agar media and incubated for further examination. After 48 h of incubation it produced small size, circular, convex colonies giving fried egg appearance with central part of colonies darker than periphery. These were confirmed by colony morphology (fried egg appearance colonies), Giemsa staining and digitonin sensitivity test (Kumar, 2000). Slide agglutination test with mycoplasma isolate developed agglutination within one to two minutes. Double immune diffusion with standard sera also produced the line of precipitation after 24 h. These two confirmed the mycoplasma species as M. bovis.

Postmortem always help in finding the cause of death (Al-Qudah et al., 2008; Almalaik et al., 2008). Necropsy showed the presence of both macroscopical and microscopical lung lesions involving the apical and cardiac lobes to the extent of 20-40% of lung area. As M. bovis settles down predominantly in the broncho-alveolar region in the respiratory tract the histopathological examination revealed lesions comprised of interstitial pneumonia (Fig. 1) accompanied by perivascular and peribronchial lymphoid cell infiltration (Fig. 2). Accumulation of desquamated cells
and serous exudate in bronchioles (Fig. 2) and alveoli with catarrhal pneumonia and the presence of inflammatory foci encapsulated by connective/fibrous tissues (Fig. 3) were also observed. These findings of postmortem were comparable to the findings reported by Rodriguez et al. (1996).

*M. bovis* infection occurs throughout the world (Kumar et al., 2011). It has also been associated with meningitis in calves (Stipkovits et al., 1993), decubital abscesses (Kinde et al., 1993), keratoconjunctivitis (Kirby and Nicholas, 1996; Pfutzner and Sachse, 1996) and otitis media (Walz et al., 1997). In a survey conducted by the Office Internationale d’Epizooties (OIE) in over 48 countries, *M. bovis* was a major component of the calf pneumonia complex, with isolation rates of 23 to 35% (Nicholas et al., 2000). The present study also established *M. bovis* as major and potent cause of morbidity and mortality in two sheep flocks. In Britain alone losses in calves are estimated to be as much as £50 million per year from mortality and costs of treatment (Rebhun et al., 1995).

The outbreak occurred in the end of rainy season during August 2010. Although the sheep were also showing the symptoms of pneumonia but most of the mortality occurred in younger animals and 12 sheep died within the period of one week in spite of continuous treatment. Clinical signs of pneumonia were evident in almost all the animals suffering from disease. The infected animals showed pronounced clinical signs; depression, dyspnoea, respiratory distress, nasal discharge, coughing and pyrexia with progressive emaciation in almost all the cases examined. Few lambs had abundant mucoid to purulent nasal discharge, infrequent lacrimation, scouring or conjunctival congestion and symptoms of pneumonia along with incoordination and inability to rise and move in advance stage ultimately leading to death. As respiratory tract infections in sheep, especially pneumonia continues to be an important cause of economic loss (Tibbo et al., 2001; Al-Qudah et al., 2008). Most of the time respiratory disease is multifactorial in sheep (Lacasta et al., 2008) and a number of causative agents were responsible for the respiratory disease complex including mycoplasma infections. Progress in understanding the pathogenesis of pneumonia has been slow because of its complex etiology and varied epidemiology (Woldemeskel et al., 2002). Therefore, for confirmation of etiological agents gross and histopathological examinations were conducted along with isolation and confirmation of organism.

Further *M. bovis* is considered the mycoplasma of relative high resistance under some environmental conditions (Nagatomo et al., 2001) hence, the role of factors such as the litter, tools and the hands and clothing of the owners and handlers might play role in spreading of the infection. Thus the identification and timely treatment is precious.
The main mode of transmission aerosol route as the infected animals releases a large amount of small, inhaled droplets during coughing. Moreover, infections of *M. bovis* are difficult to treat (Stalheim, 1976) as most *M. bovis* strains are often resistant to antibiotics (Ball *et al.*, 1995; Thomas *et al.*, 2002). Although, Hannan (2000) recommended that antibiotic susceptibility of the isolates can only be examined by the determination of the Minimal Inhibitory Concentration (MIC) values instead the disc diffusion test but this method can be used for early recommendation of drug of choice and could prevent economic losses. Thus antibiogram of the organism was conducted using the disc diffusion method, *M. bovis* isolates was resistant to almost all the commonly used antibiotics except Tylosin and Enrofloxacin. Ciprofloxacin and Tiamulin showed intermediate response whereas other antibiotics were of no use. Depending upon the zone of inhibition Tylosin was the drug of choice and recommended for the treatment of flock. The findings of present study regarding the drug sensitivity patterns are in the concurrence to the findings of many other workers as according to Aylng *et al.* (2000) and Nicholas *et al.*, (2000) all strains of *M. bovis* had developed resistance to tilmicosin, most were found to be resistant to oxytetracycline and about 20% were resistant to spectinomycin and florfenicol. Only danofloxacin was effective *in vitro* against all strains. All of the mycoplasmas are resistant to beta-lactames because of the lack of the cell wall. Poumarat *et al.* (1994) observed resistance against nalidixic acid, polymyxin, rifamycin, trimethoprim and to sulfonamides. Initially tetracyclines, macrolids and aminoglycosides were used with good results against *M. bovis* but later on many resistant strains had been reported (Ball *et al.*, 1995; Aylng *et al.*, 2000). Similarly the antibiotics of these groups were found resistant and revealed no or very small zone of inhibition. Most of the mycoplasmas including *M. bovis* are sensitive to antibiotics which inhibit the protein or nucleic acid synthesis. The most effective antibiotics are thepleuromutilins (tiamulin, valnemulin) and the fluoroquinolones (Taylor-Robinson and Bebear, 1997; Thomas *et al.*, 2003). Tiamulin has been reported with excellent activity against *M. bovis* (Ter Laak *et al.*, 1993; Friis and Szczver, 1994; Hannan *et al.*, 1997). In contrast to these, the *M. bovis* isolate showed intermediate sensitivity however, similar to the findings of others (Ball *et al.*, 1996; Hannan *et al.*, 1997; Aylng *et al.*, 2000) enrofloxacin was also found effective against the *M. bovis* isolate *in vitro* but some workers do not recommend their use in the practice, because of their failure in diminishing respiratory losses (Nicholas and Aylng, 2003). Therefore, Tylosin was preferred over Enrofloxacin for the treatment of infected flock. The disease conditions induced by *M. bovis* were successfully treated with tylosin. Significant improvement of clinical signs was observed within 3-5 days after the start of medication and clinical signs disappeared within one week. Medication restored the animals’ appetite and weight gain approached to the healthy sheep within month and no animal revealed post treatment *M. bovis* in nasal and tracheal swabs.

**CONCLUSION**

Mycoplasmosis is a common problem in sheep particularly in meditarian countries where it causes severe economic losses. In India there has not been much attention paid to respiratory diseases due to mycoplasma infection. However, many reports of mycoplasma isolation from sheep have been reported from various parts of country time to time. This study appeared to be first of its kind for the isolation of *M. bovis* from the outbreak in sheep flock leading to mortality and high morbidity rate. As mycoplasma differ from cellular composition from bacteria so commonly used antibiotics mostly do not cure them. In this study, tylosin appeared to be the drug of choice and its application cured the flock.
REFERENCES


