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Isolation and identification of mycoplasma from respiratory system of goat

Md. Humayun Kabir* and A. S. Mahfuzul Bari

Department of Pathology, Bangladesh Agricultural University, Mymensing, Bangladesh.

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ABSTRACT

In Bangladesh there is no published report of mycoplasmosis in Black Bengal goats. In the present study, attempts have been undertaken to investigate the clinical samples for the isolation and identification of *Mycoplasma* in goats. In addition, the morphological changes (gross and histopathology) of mycoplasmosis in naturally infected Black Bengal goats and morbidity and mortality rates of mycoplasmosis in goat were also studied. A total of 100 samples, lung exudates, swabs from trachea, nasal cavity and suspected lungs from the slaughter houses were studied. Out of the 100 samples, 27 samples were clinically suspected cases from the USDA funded research goat farm (USDARGF) at the artificial insemination center of BAU and 10 clinically healthy goat's samples. The rest 63 samples were taken from municipality slaughter houses in Mymensingh district during July to October, 2010. Morbidity and mortality rate of suspected mycoplasmosis in USDARGF were 32.14 and 15%, respectively. Samples were collected in 10% formalin for histopathological study. Swabs were collected in *Mycoplasma* broth supplemented with horse serum and Kanamycin solution for the isolation of pathogenic goat mycoplasmas. Additionally, Kanamycin solution was added to prevent the growth of gram –ve bacteria. In this study, "possible etiological agent" *Mycoplasma* was investigated for the first time and found that 8% cases were positive for mycoplasmosis as confirmed by isolation and identification of colony characteristics. The yellowish foci, pea sized nodule and fibrin deposit were in the lung surface. The pulmonary pleura become thickened, fibrin deposit and there were adhesions to the chest wall. In microscopic field the lungs showed bronchopneumonia, fibro-purulent pneumonia with adhesion pleuritis and thickening of the alveolar walls. The *Mycoplasma* spp. were identified from colony with Gram's staining and Giemsa staining. Moreover, in this study the organisms were also directly demonstrated in the tissue by the mentioned staining. It seemed from the study that mycoplasmosis is the common disease of the respiratory tract of goats. However, further studies could be carried out to confirm the *Mycoplasma* by Polymerase Chain Reaction (PCR).

Keywords: Black Bengal goat, pneumonia, bronchopneumonia, fibropurulent pneumonia, *Mycoplasma* spp., culture.

*Corresponding author. E-mail: hkabirvet@yahoo.com.

INTRODUCTION

The Mycoplasma respiratory disease has a special place in veterinary medicine (Stalheim, 1983). The goat is kept as a source of meat, milk, and fiber. Often described as the "poor man's cow," the goat can survive in areas, where a cow cannot and therefore, replaces the cow in importance for a large portion of the world's population. Improve goat husbandry helps to maximize human food supplies from marginal agricultural lands under restrictive climatologic circumstances (DaMassa et al., 1992). Among the important goat diseases, mycoplasmal infections result in significant losses for the African continent and in countries such as Greece and France (Cottew et al., 1974; Emo et al., 1978). Mycoplasmas are the smallest fastidious bacteria which can cause diseases in major species of animals including humans. Several Mycoplasma species cause serious and economically important diseases in goats world-wide. Mycoplasmas have a specific attachment among the

mucociliary system of the bronchioles (Cheville, 1983). The economic losses associated with the disease are often the result of a complex interaction between infection, poor management and environment condition (Daniel et al., 2006). In small ruminants, they are known for respiratory disease, arthritis, conjunctivitis, genital disease and mastitis (Awan et al., 2009). Most of the members of *Mycoplasma mycoides* cluster group are the important pathogens of small ruminants. This group comprises six species and subspecies. Some of these mycolasmal species can cause severe and contagious diseases in goats with significant economic impact (Cottew and Yeats, 1978). Of the many mycolasmal diseases, contagious caprine pleuropneumonia (CCPP) is a highly fatal disease that occurs in Eastern Europe, the Middle East, Africa and Asia (Awan et al., 2009).

It is caused by *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp). This organism is closely related to three other mycoplasmas; *M. mycoides* subsp. *mycoides* large colonies (LC), *M. mycoides* subsp. *capri*, and *M. capricolum* subsp. *capricolum*. Unlike true CCPP, which is confined to the thoracic cavity, the disease caused by the latter three mycoplasmas is accompanied by prominent lesions in other organs and/or parts of the body besides the thoracic cavity. It is a significant disease of goats in Africa, the Middle East and Western Asia, with mortality rate up to 90% the susceptible herds. Clinical disease has so far been reported in 38 countries, with 11 countries having isolated the causative organism (Thiaucourt and Bloske, 1996).

Mycoplasmal diseases may not be diagnosed solely on the basis of clinical signs, pathological lesions or serological tests because of the close association between the *Mycoplasma* organisms. Isolation and identification are, therefore, required to confirm diagnosis, but this requires a specialized laboratory with experience of these very fastidious organisms. The classical methods for detecting and identifying mycoplasmas are time consuming and complicated by serological cross reactions between the closely related organisms.

In this study, an attempt has been undertaken to isolate the *Mycoplasma* from randomly samples of goat from the USDARGF at the Artificial insemination center of BAU and the suspected clinical samples from the Municipality slaughter house, Mymensingh.

MATERIALS AND METHODS

The research work was conducted in the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. A total of 84 goats exist in USDARGF at the Artificial Insemination (AI) center of BAU. The clinically affected cases were 27 and from affected cases, death occurred in 4 goats. In this study, a total of 100 samples; lung exudates, swabs from trachea, nasal cavity and suspected lungs from slaughter houses were studied. Out of these 100 samples, 27 samples were clinically suspected cases from the USDARGF and 10 clinically healthy goats' samples. The rest 63 samples were from Municipality slaughter houses, Mymensingh.

All the samples were subjected for isolation and identification of Mycoplasma. The collected tissues were fixed in 10% formalin and processed for routine histopathological examination. Study of morbidity and mortality rates of the respiratory mycoplasmal disease in goat was performed.

RESULTS

Morbidity and mortality

In this study, morbidity and mortality rates of mycoplasmosis in USDARGF were 32.14 and 15%, respectively. Clinical cases were recognized by clinical symptoms such as nasal discharge, inappetence, coughing, dyspnea, pyrexia and sometimes lameness.

Gross lesions

Catarrhal exudates in the nasal passages and trachea were observed. In some cases, hemorrhages were also present in the trachea. The yellowish foci and fibrin deposit on the surface of the lungs (Figure 1). The pulmonary pleura becomes thickened, fibrin deposit and there were adhesions to the chest wall (Figure 2). Pea sized yellowish nodules were seen in the lungs in some cases (Figure 3), there were marked congestion around the nodules with somewhat solidified. The lesions may be confined to one lung or involve both, and an entire lobe may become solidified.

Cultural characteristics

Out of 100 samples, only 8 samples were found to be positive in culture, confirmed by typical colony characteristics and staining properties by Gram's staining and Giemsa staining.

Out of the 27 suspected samples from the USDARGF, only one was positive. The rest 7 positive samples were found from the slaughter house samples. The 10 clinically healthy samples from the research goat farm were negative.

The activities and the changes found during progressive growth of *Mycoplasma* in broth and agar with horse serum are presented in the Tables 1 and 2. After seven days formation of turbidity in the test tubes was considered positive in primary culture.

Mycoplasma agar showed growth of organisms as numerous tiny colonies that could be found after 4 days (Figure 4). In the second passage at day 8 off white color colonies became enlarged in size and formation of the slight dense area in the centre center looking fried egg appearance (Figure 5).

Gram's staining

After primary culture of the organism in Mycoplasma

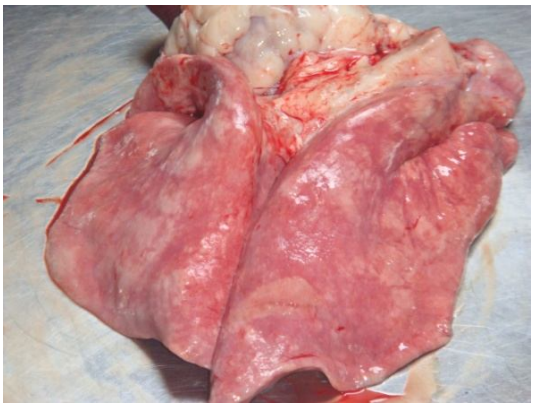

Figure 1. The yellowish foci and fibrinous deposit on the surface of the lungs in *Mycoplasma* infected goat.

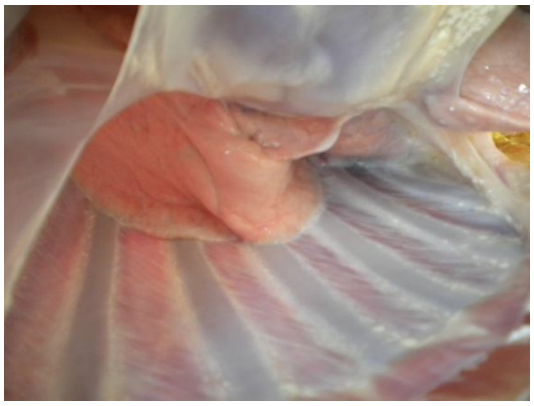

Figure 2. The yellowish, fibrinous deposit on the surface of the lungs, thickening of pleura and adhesions to the inside of the rib cage in *Mycoplasma* infected goat.

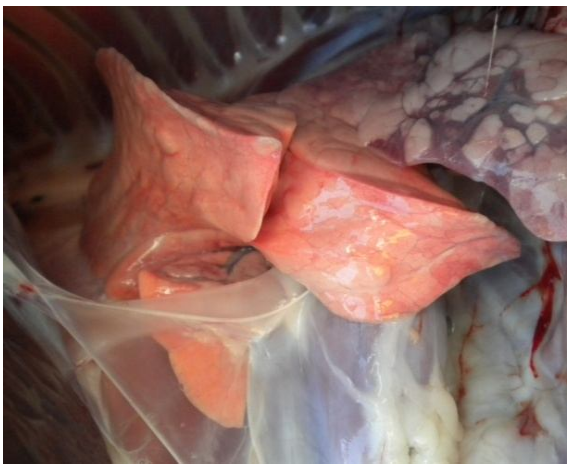

Figure 3. Consolidated lungs, the yellowish pea form nodules and fibrinous deposition on the surface of the lungs with adhesions to the inside of the rib cage in Mycoplasma infected lungs.

agar, Gram's staining was done to have a preliminary idea about the organism. In Gram's staining under microscope, the organisms revealed weakly gram-negative, pink color and coccoid in appearance.

Giemsa staining

The organism was stained well with Giemsa stain. In Giemsa staining under microscope, the organisms revealed coccoid, flask- shaved polarity of the cell body or filamentous in appearance.

Staining (lung sections)

Microscopy of lung sections was showed *Mycoplasma* spp. as coccoid morphology in both Gram's staining and Giemsa staining.

Histopathological study

In microscopic field the lungs showed bronchopneumonia characterized by filling of bronchus with exudates (Figure 6). In some area fibrinous inflammation is predominant (Figure 7) with adhesion pleuritis causing fibropurulent pneumonia. The alveolar wall was thickened due to increased number of cells, alveolar space became small compared to normal alveolar wall (Figure 8). Many inflammatory cells were observed in these areas of the lung (Figure 9). The *Mycoplasmae* were also demonstrated in the lung section with Grams staining and Giemsa staining which were looking as coccoid morphology.

Morbidity and mortality rates of mycoplasmosis in USDARGF were 32.14 and 15%, respectively. In this study an attempt has been undertaken to isolate the Mycoplasma from the Black Bengal goat and their gross and histopathological studies from naturally infected cases. Mycoplasmosis in goat is common in African countries (Cottew et al., 1974; Emo et al., 1978) but there was no published report of mycoplasmosis in Black Bengal goat in Bangladesh. The clinical signs of mycoplasmosis include nasal discharge, coughing, dyspnea, pyrexia etc (Kusiluka et al., 2007). These signs may easily confuse with other respiratory diseases like bacterial pneumonia, lung worm, common cold, etc. In this study, possible etiological agent as Mycoplasma was investigated for the first time and found that 8% cases were positive for mycoplasmosis confirmed by isolation and identification of colony characteristics.

The one of the main objectives was to surveillance of goat mycoplasmosis in the USDARGF at BAU. During the study period only 27 cases were suspected goat mycoplasmosis in the research goat farm, however, only one was positive by isolation and identification. The rest

Table 1. Activities and changes found during the progressive growth of Mycoplasma in broth.

Date and duration of observation	Activities and changes found during the progressive growth of *Mycoplasma* in broth
At day 1	In Mycoplasma broth: Collected samples such as nasal, tracheal and lung swabs were taken in the screw cap test tube containing Mycoplasma broth. Then it was placed in incubator for incubation at 37°C.
After 7 days	After seven days formation of turbidity in the screw cap test tube. Formation of turbidity indicates growth of *Mycoplasma* within the broth. As it shows turbidity, we allowed it for subculture in plate with Mycoplasma agar kept in a candle jar with sufficient CO_2 tension and moisture content.

Table 2. Activities and changes found during the progressive growth of Mycoplasma in mycoplasma agar using horse serum.

Date and duration of observation	The changes found during the progressive growth of Mycoplasma in Mycoplasma agar using horse serum
At day 2 days	Off white colored growth of Mycoplasma colony. Formation of numerous single colony, no elevated area and completely homogenous.
After 4 days and 6 days	Off white color colonies. In all of the colonies slight concentrated area was shown at the center. On the same day 2nd passage was given to another Mycoplasmal agar plate (Prepared by using Horse serum) from the concentrated area. In the 2nd passage at day 4th off white colored growth of Mycoplasma and a number of colonies were formed.
After 8 days and 12 days	Off white colored and colonies became enlarged in size and formation of a slight dense area at the center of all colonies. In the 2nd passage at day 8th center of all colonies were more dense and concentrated and elevated that seems to fried egg appearance.

Figure 4. Mycoplasma agar showing growth of organisms as numerous tiny that could be found after 4 days in agar media.

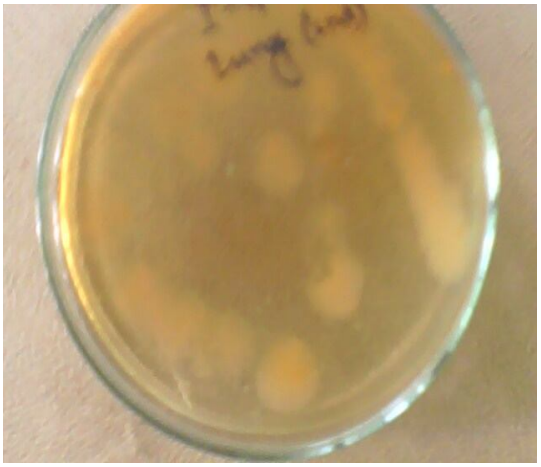

Figure 5. Mycoplasma agar showing growth of organisms which is typical fried egg appearance that could be found in the 2nd passage at day 8.

26 cases were negative. They might be due to bacterial or viral cases of respiratory symptoms. However, they recovered after antibiotic therapy, but did not attempt to isolate the bacteria or virus.

This study indicates that goat mycoplasmosis is present in low percentage and USDARGF has also low incidence. Improvement of biosecurity of the farm might improve the condition not only of mycoplasmosis but also other infectious diseases.

In this study, routine methods of mycoplasmal cultures in Mycoplasma selective media with kanamycin were used for the isolation of *Mycoplasma* spp. In this study, kanamycin solution was used in broth media to overcome the problem because it prevents the growth of Gram

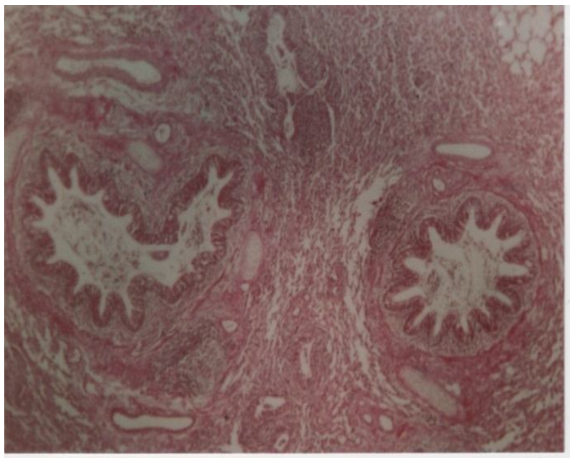

Figure 6. Showing bronchopneumonia characterized by filling of bronchus with exudates in the lung tissues (H & E staining × 104).

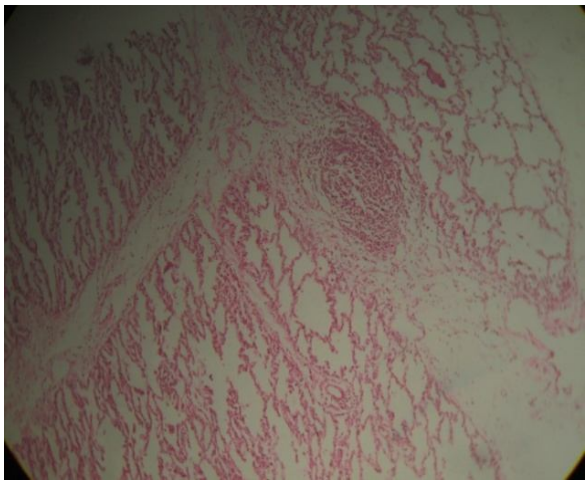

Figure 7. Showing fibropurulent pneumonia in lung tissues with deposition of fibrin (H & E staining × 100).

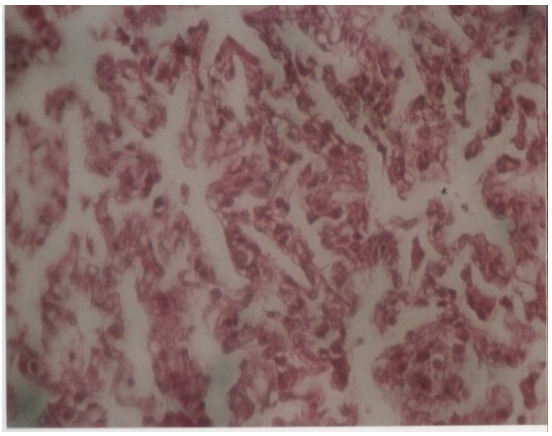

Figure 8. Showing the alveolar wall was thickened due to increased number of cell alveolar space became small in the lung tissus (H & E staining × 260).

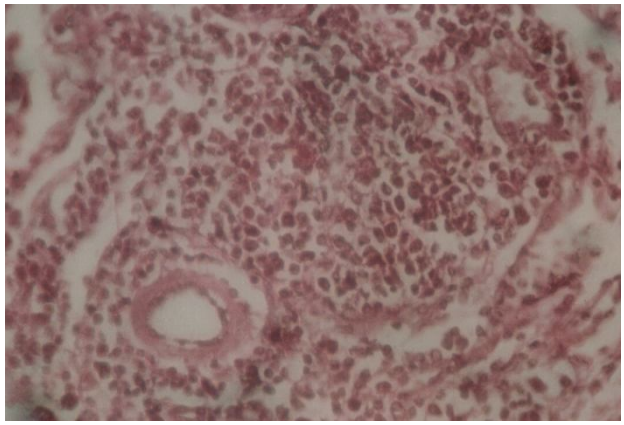

Figure 9. Inflammatory cell infiltrations were observed in bronchus in the lung (H & E staining × 260).

negative bacteria. Samples were collected from nasal, trachea and lung in this study.

Several suitable culture media have been formulated (Freundt, 1983) and medium suitable for isolation of goat mycoplasmas such as Frey's medium or a modification Frey's medium (Kleven, 1994), and other authors supported Mycoplasma agar base, Mycoplasma broth base, Mycoplasma supplement–G (Oxoid Manual, 1998, E.Y. Bridson, Basingstoke, Hampshire, England) or horse serum.

After collection of samples, the swabs were inoculated into Mycoplasma broths and incubated at 37°C for 7 days which were supported by different authors (Hirsh and Chung, 1999; Ezzi1 et al., 2007; Ley and Yoder, 1997; Harasawa et al., 2004; Gharaibeh and Roussan, 2008). Turbidity was found in the mycoplasmal broth and the results are in agreement with a previous study (Del and Tully, 1995; Kleven, 1997; OIE, 2008).

Most *Mycoplasma spp.* grow best at 37°C, increased humidity, and CO_2 tension in the atmosphere to enhance growth of *Mycoplasma* (OIE, 2005).

Mycoplasma cultured on PPLO broth media 3 to 7 days incubating at 37°C and then across the surface of an agar plate before the colonies were apparent. The positive colonies were examined every 48 h. After 72 h the first positive cases were confirmed (Hirsh and Chung, 1999). Growth of the organisms was observed after 7 days, but typical colonies were found after 19 days in Mycoplasma agar which were smooth, circular, fried egg appearance colony and there were no growth of bacteria due to use of the Kanamycin solution in broth corresponded with the findings of other authors (Del and Tully, 1995; Yoder and Hofstad, 1964; OIE, 2008).

In microscopy of lung sections *Mycoplasma* spp. showed a coccoid or short filamentous morphology although which does not provides a definitive diagnosis,

but it is similar to other finding (OIE, 2008).

Considering all of the constraints and the scarcity of available methods for identification, a specific polymerase chain reaction was developed (Woubit et al., 2004). Polymerase Chain Reaction (PCR) studies were confirmed the genus of Mycoplasma although attempting for identification of strains *Mycoplasma mycoides*, *Mycoplasma capricolum*/Caprine pleuropneumonia and *Mycoplasma arginin* were in failure (Ezzi et al., 2007).

Polymerase Chain Reaction (PCR) is a molecular technique which is specific for identification and differentiation of *Mycoplasma spp.* (Slavik et al., 1993; Silveira et al., 1996; Zhang et al., 1999; Lia et al., 2000).

In this study, lungs were solidified which is supported by another finding (Hutchison and Montague, 2002).

In the present study, the histopathological characteristic of *Mycoplasma* in goat is similar to other finding (Ezzi et al., 2007; Hutchison and Montague, 2002; Thigpen et al., 1981).

Moreover, in this study the organisms were also directly demonstrated in the tissue by the and staining. This finding strongly supported the isolation from the tissue. However, further studies are needed to confirm *Mycoplasma* by Polymerase Chain Reaction (PCR).

In summary, typical *Mycoplasma* colonies were found after 2nd passages of 19 days inoculation period. Other bacterial growth was not demonstrated due to the addition of kanamycin solution in the Mycoplasma broth. However, bacterial contamination is a common problem encountered in Mycoplasma isolation. This method might be useful for many laboratories, because the antisera are not commercially available which is required for serological tests. In this study, preliminary results from field samples suggest that culture using kanamycin solution could be a useful diagnostic test for the isolation of pathogenic goat Mycoplasmas.

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