Pathological description of naturally occurring *Mycoplasma bovis* associated pneumonia in bovine calves

Pankaj Goswami*, Harmanjit Sing Banga and Vishal Mahajan

Department of Veterinary Pathology, College of Veterinary Science, GADVASU, Ludhiana-141 004, Punjab, India.

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**ABSTRACT**

*Mycoplasma bovis* is an important etiologic agent of bovine respiratory infection and predominantly causing chronic pneumonia lesion. The present study encompassed standardized pathological, immunohistological and molecular technique for diagnosing natural bovine pneumonic cases with *Mycoplasma bovis* associated lesions. During the study, examination of 87 carcasses putatively died of pneumonia where *Mycoplasma bovis* was ascertained in two bovine female calves by immunohistological staining and Polymerase chain reaction. The specific pathological lesions were noted as chronic bronchopneumonia with mild to moderate caseonecrotic lesion and suppurative bronchopneumonia typical of *Pasteurella* infection. The other bacterial organism associated with these cases was found to be of *Pasteurella multocida* detected on isolation. No viral etiological association was detected in immunohistological examination in respect of bovine parainfluenza type 3, bovine respiratory syncytial virus and infectious bovine rhinotracheitis virus. The result also revealed that *M. bovis* infection may develop into a severe necrosuppurative bronchopneumonia when associated with high numbers of intraleisional organism and into milder catarrhal bronchointerstitial pneumonia when associated with low numbers of organism.

**Key words**: Bovine, Histopathology, Immunohistocemistry, *Mycoplasma bovis*, Pneumonia, PCR.

**INTRODUCTION**

Bovine respiratory infection is one of the complexes etiological entities with appreciable clinical significance and mortality. Several factors such as environmental as well as concurrent viral and bacterial infection are incriminated for developing pneumonic lesions (Cusack et al., 2003). *Mycoplasma bovis* is an important pathogenic agent causing predominantly chronic pneumonia and arthritis in calves and young cattle and mastitis in adult cattle. Both in spontaneous natural and experimentally infected animals, different pattern of inflammatory lung lesions occur, among which caseonecrotic is considered distinctive for Mycoplasmal infection (Caswell and Archambault, 2008; Caswell et al., 2010). Mycoplasma species has been incriminated with increase severity of respiratory disease in calves and can also act as primary pathogen (Gagea et al., 2006). *M. bovis* is more accepted as a cause of chronic bronchopneumonia with caseous and perhaps coagulative necrosis, characterized by persistent infection that seems poorly responsive to many antibiotics (Caswell and Archambault, 2008). Retrospective analysis of archival material has revealed that characteristic lesion were overlooked or attributed to other pathogens (Gagea et al., 2006) and it is difficult to diagnose clinically in differentiating pneumonia attributable to infection with *M. bovis* from other forms of bacterial pneumonia, unless concurrent polyarthritis is present. Although the role of *Mycoplasma bovis* in the bovine pneumonia is generally recognised, there is scanty report from India about the occurrence of *Mycoplasma* in bovine. Therefore, the aim of the present study was a more detailed investigation of occurrence of *Mycoplasma bovis* in the respiratory infection of cattle and the association of mycoplasma with other common etiological agents like virus or bacteria.

**MATERIALS AND METHODS**

Gross and microscopic examination was performed in addition to immunohistological and molecular diagnosis. In this study 87 bovine carcasses showing visible pneumonia lesion at necropsy were studied and distribution of pneumonic lesions were recorded. The lung tissue sample collected and subjected for diagnosis of Mycoplasma spp by isolation, immunohistochemical and PCR technique. The lung samples from areas with inflammation and from normal areas were taken for histopathological examination and infections identified were characterized using standardized scoring system as laid by Booker et al (2008) with some modification.

Lung samples for the isolation of Mycoplasma were cultured according to the method described by Ter Laak et al (1992) and Radaelli et al (2008). Swab and lung sample were collected aseptically on PPLO (pleuropneumonia like organism) broth tubes during at postmortem and incubated at 37°C for 5 days. For Mycoplasma, slight colour change
of broth media due to pH shift was examined daily. The broth cultures were transferred after 5 days onto solid medium (Mycoplasma agar base, HiMedia lab) and incubated at 37°C in 5% CO₂ atmosphere for another 5 days. The broth and agar medium both were supplemented with 25% horse serum (HiMedia lab) during preparation. The plates were examined under stereomicroscope and searched for presumptive identification of characteristic nipple shaped or fried egg colonies of Mycoplasma spp. The bacterial isolation also tried with lung homogenates for Pasteurella spp. Lungs tissue homogenates were made (1:10) and initial culture were brought on 5% sheep blood agar by conventional streaking method and were incubated at 37°C for 24-48 hours. The growth on blood agar then sub cultured on Bovine heart infusion (BHI) agar and Macknokeys’ Lactose Agar (MLA). The isolates were then identified as Pasteurella multocida on the basis of morphological criteria and biochemical test as described previously (Dousse et al. 2008). A first reading was taken at 24 hour followed by a second reading at 48 hours to check the proper growth of organism. The isolate recovered were sub-cultured for getting pure culture and identified using colony morphology, detection of haemolysis, Grams stain procedure, Methylene blue staining and biochemical tests. The biochemical tests employed were Indole formation, Glucose, Lactose reaction, Nitrate reduction test in Hi-assorted Biochemical Test kit from Hi Media laboratories and oxidase and catalase production test (Quinn et al 2002 and Dousse et al 2008).

Histopathological tissue sections of lung were subjected for Mycoplasma infection were confirmed by localization of antigen in tissues using Mycoplasma bovis specific monoclonal antibody. The commercially available antibody against Mycoplasma bovis raised on Mouse was used (MY11-320.1, ab62385 from Abcam). For immunohistochemical studies 4-5 µ thick paraffin-embedded sections were prepared routinely. Sections were deparaffinized in 2 changes of a clearing agent, 15 min each, and rehydrated were prepared for the tissue section in 10mM Sodium citrate buffer at a temperature of 95°C for 10 minutes followed by 98°C for 5 minute using EZ—Retriever System (BioGenex Laboratories Inc., San Ramon, California, USA). Following HIER, the sections were allowed to cool to room temperature and given three washing in Phosphate Buf fer Saline- tween (PBST) solution (pH 7.4) for three minutes each. The activity of non specific proteins was blocked by incubating both the sections in ready to use Horse serum (Vector Laboratories, Burlingame, California, USA) for 15 minutes at room temperature in a humidified chamber. Thereafter draining the blocking serum, targeted sections were incubated overnight at 4°C with reconstituted primary antibody (anti Mycoplasma bovis antibody 1:100) and the other section with PBS without primary antibody as negative control. Then the sections were given washing in triplicate with PBST (pH7.4) of 3 min each. Following this section were incubated with Polymer HRP (Bio Genex Laboratories, Inc., San Ramon, California, USA) for 30 minutes in a humidified chamber and given three washing with PBST for 3 minutes each. The antigen-antibody—peroxidase reaction was visualized following the manufacture’s instruction, 3’ 3’ Diaminobenzidine tetrachloride (DAB) solution (Vector Laboratories, Burlingame, California, USA) prepared freshly and poured over the section for 40 seconds or till the development of light brown reaction, which ever was earlier. The reaction was stopped by rinsing slides in distilled water for 5 minutes and counterstained with Gill’s Haematoxylin (Merk, Germany) for 30 seconds and washed in running tap water for 5 minutes. Finally, the slides were dehydrated in ascending grades of alcohol, cleared in xylene. A brownish colour (the oxidized, peroxidase-developed chromogen precipitate) observed by light microscopy within the tissue sections on sample slides was interpreted as a positive IHC reaction. Control sections incubated with PBS instead of primary specific antibody were found to lack IHC staining. Involvement of other viral infection like Para influenza Virus -3 (PIV3), Bovine Respiratory Syncytial Virus (BRSV) and Infectious Bovine Rhinotracheitis (IBR) were also screened in lung sample by immunohistochemistry technique as described for Mycoplasma bovis. However for PIV3, BRSV and IBR, commercially available primary antibody were used as mouse monoclonal PIV3 (Abcam, ab64551, 1:100), rabbit polyclonal BRSV (Abcam ab3478; 1:100) and Mouse

Table 1: Histopathological score of different pathological process within the two individual positive cases with immunohistochemical score ++.

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<tr>
<th>Case no</th>
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<th>SB</th>
<th>BI</th>
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FB—fibrinous bronchopneumonia  
SB—suppurative bronchopneumonia  
BI—bronchointerstitial pneumonia  
PL—pleuritis  
IP—interstitial pneumonia  
FNP—fibrinonecrotizing pneumonia  
NP—caseonecrotic pneumonia  
VC—vascular compromise

Scoring system for each pathologic process was: 0—none, 1—focal or multifocal lesion, 2—single locally extensive lesion, 3—widespread extensive, or multiple locally extensive or diffuse lesion.
monoclonal BoHV (BMRD 1:50) respectively. For Mycoplasma antigen detection IHC staining of tissue was scored as 0, +, ++, +++ scale indicating negative staining. Small sized foci, (< 50% of the area of fields is comprised of tissue that is positively stained for antigen in 200 x), Medium sized foci,( < 50% of the area of the fields are comprised of tissue that is positively stained for antigen in 200x ) and Large sized foci, ( >50% of the area of most fields is comprised of tissue that is positively stained for antigen in 20x), respectively.

To confirm the presence of mollicutes class, polymerase chain reaction (PCR) was used. PCR amplification was carried out to detect Mycoplasma species following extraction of DNA from lung samples. The genus specific forward and reverse primer, GPO-3 (5´-GGGAGCAACAGGATTAGATACCT-3´) and MSGO (5´-TGCACCATCTGTCACTCTGTTAACCTC-3´) respectively as suggested by Van Kuppeveld et al (1994) were employed for expected product size of 280bp.

**RESULTS AND DISCUSSION**

In this study two cases of pneumonia of chronic nature were positive for Mycoplasma bovis by immunohistological staining and polymerase chain reaction. The approximate age of the positive animals were 2.5 month and around one year.

Isolation of Mycoplasma bovis was attempted from lung homogenates but none of the sample showed cultural growth specific for Mycoplasma spp. The Mycoplasma positive sample were also subjected for bacterial isolation of Pasteurella spp and revealed one sample positive and another sample showed multibacteria overgrowth missed the specific results. Tegtmeier et al., (2000) opined that bacterial culture for Mycoplasma spp have the lowest sensitivity when compared to IHC or PCR techniques. One reason for this difference may be that to culture bacteria the presence of viable bacteria is a requirement. In the present study, negative culture of Mycoplasma spp might be related to viability of organism or contamination of sample during collection process leads overgrowth of multibacteria masking the Pasteurella organism.

**Pathological examination:** In the present study, pneumonic lesions caused by Mycoplasma bovis had a cranioventral distribution with relative sparing of caudal and dorsal aspect of caudal lobes. Consolidation and necrosis of lung parenchyma were found grossly. The observations seen in this study are similar to those reported by Caswell et al. (2010). Lesions in the cranioventral lobes covered around 80% of lung parenchyma with formation of multifocal abscess and necrotic areas (Fig.1a). The affected areas of lung appeared collapsed, congested or haemorrhagic. In one case, the 50% of the lungs appeared red, collapsed and consolidated with presence of small necrotic areas without abscessation. On cut surfaces, white exudate drained off from small bronchi of cranial lung lobes (Fig.1b). Cut surface of varying abscesses (few mm to cms) were found to be inspissated. Mycoplasma associated abscess in lung was reported previously in natural cases without bacterial involvement (Kinde et al, 1993; Adegboye et al 1995). Abscessation seen in present case might have been ensued Mycoplasma bovis infection or other secondary bacterial invasion. Pasteurella sp. organism was also isolated. Besides lung lesion(s), other lesions noted were viz. enlargement of pulmonary lymphnodes (Fig. 1c), fibrinous pericarditis and mild hepatomegaly.

**Histopathological examination:** The detailed histological examination of the lung lesion revealed caseous necrotic areas developed in airways, in alveoli, or in interlobular septa with outlines of necrotic to non-necrotic leukocytes predominantly neutrophils in two animals. Distinguished abscess formation with fluid purulent centre, coagulation necrosis was also observed in few areas involving alveoli(Fig. 2a & 2b). The histological lesion was also characterized by bronchointersitial pneumonia with infiltration of neutrophils and mononuclear cell in alveoli and bronchiole in most of the cases (Fig. 2c & 2d). Pleuritis were also noted accompanying marked congestion and neutrophilic infiltration (Fig.2e). In addition, moderate numbers of multinucleated giant cells in alveolar spaces with 5-6 nuclei were found. A mixture of desquamated epithelial cells, macrophages and mononuclear cell were present in the lumen of bronchioles. In the bronchiole there were hyperplasia and
metaplasia of goblet cell along with increase mucus exudate in alveolar septa as compared to unaffected bronchiole (Fig. 2f & 2g). Goblet cell metaplasia and hyperplasia in bronchiole in induced *Mycoplasma bovis* infection also reported by Jones *et al* (1975) and Wawegama *et al* (2012) in cattle. Goblet cells are the first line of defense in the airway, producing mucus to trap and remove foreign substances from the airways. Changes to goblet cell number and distribution are features of host reaction to a local irritant *M. bovis* in this case. Similar to our findings caseonecrotic pneumonia in combination with broncho-interstitial pneumonia, suppurative bronchopneumonia, suppurative bronchitis, bronchiolitis were recorded by Hermeyer *et al* (2012a) in bovine calves on experimental intra tracheal inoculation of *Mycoplasma bovis*. The necrotic bronchopneumonia with abscess was found much severe than caseous necrotic area recorded in the present study. In the experimental study Hermeyer *et al* (2012a) recorded moderate to severe caseous necrosis in lung only in two calves out of 12 calves inoculated with *M. bovis*. *M. bovis* has been shown to have four distinct lung lesion patterns on post mortem evaluation; caseonecrotic bronchopneumonia, bronchopneumonia with coagulative necrotic foci, suppurative bronchopneumonia without necrosis and chronic bronchopneumonia with abscession (Caswell 2008). Others have reported *M. bovis* induce pneumonia to be characterized by a necrotizing bronchopneumonia with bronchiectasis and abscession (Shahriar *et al*. 2002; Khodakaram-Tafti and Lopez 2004). Similar to the present findings moderate to suppurative bronchointerstitial pneumonia with multinucleated giant cells of macrophage origin within the alveolar space were also observed by Hermeyer *et al* (2012b) in a neonatal calf.

**Fig 2:** Histopathology of lung section affected with *M. bovis*, (H&E).

2a: caseous necrosis with abscessation, zone of inflammatory cells (10x). 2b: purulent inflammation with necrosis (40x). 2c: Necrotic bronchopneumonia, PMNs & lymphoid cells (10x). 2d: Bronchointerstitial pneumonia (10x). 2e: Pleuritis with neutrophilic infiltration in alveolar lumen (10x). 2f: Lung: Goblet cell metaplasia in bronchiole (20x). 2g: Unaffected bronchiole with negligible goblet cell (10x). 2h: Moderate congestion and haemorrhage in pulmonary lymphnode (10x).

**Fig 3:** Immunohistochemistry staining of *M. bovis* antigen in Lung tissue section.

3a: IHC staining reaction (arrow) of *M. bovis* in periphery of necrotic area (N) (20x). 3b: Sparing IHC staining of *M. bovis* in necrotic area as well as in macrophages (40x). 3c: *M. bovis* in desquamated bronchiolar epithelium and broncho-pneumonia (40X). 3d: IHC staining (arrow) of *M. bovis* in bronchiolar epithelium in affected bronchiole with neutrophilic infiltrations (20X). 3e: IHC staining of *M. bovis* in neutrophils in alveoli (40X). 3f: Negative control tissue section for *M. bovis* (IHC 20X)
affected with *Mycoplasma bovis*. Co-infection of *M. bovis* with other pathogens including *Pasteurella multocida*, *P. haemolytica*, *A. pyogenes* and less frequently with respiratory viruses were commonly reported (Shahriar et al 2002; Gagea et al 2006 and Caswell et al 2010). The arthritis lesion tends to occur due to haemogenous spread of organism. The transmission of *M. bovis* occurs via ingestion and inhalation route to establish infection in respiratory system and via hematogenous spread to develop septicemic form or arthritis (Maunsell and Donoven, 2009). The present case of infection may be contacted through inhalation and set up lesion in lungs without septicemic spread to produce arthritis.

The histopathological lesions were not much specific in other visceral organs. The mediastinal lymphnode showed moderate congestion and haemorrhage with less population of lymphocytes in lymphoid follicle (Fig 2h). Mild hepatitis with periportal lymphocytic infiltration was noted in few cases. The cardiac lesion consisted of congestion of vessels and mild endocardial degeneration. The changes might be resulted of immunosupression and associated bacterial invasion during the disease process.

**Immunohistochemical staining of *Mycoplasma bovis***: Immunohistochemical staining of lung tissue sections for *Mycoplasma bovis* revealed positive immunoreactions in two cases (18.18%). Immunolabelling of *M. bovis* antigen was demonstrated in bronchial epithelial cells and in leucocytes and cell debris of the airways (Fig 3). Abundant granular immunoreaction was located predominantly at the periphery of foci of caseous necrosis (Fig.3a&3b) *M. bovis* is typically found at the margins of the necrotic areas but is also found within macrophages and bronchiolar exudates (Rodriguez et al 1996; Shahriar et al 2002; Khodakaram-Tafti and Lopez 2004; Gagea et al. 2006 and Caswell 2008). Immunoreactivity was also associated with the necro-suppurative exudate plugging bronchiolar lumina and was occasionally observed along the bronchiolar epithelia cells surface, as well as cytoplasm of neutrophils(Fig. 3c, 3d&3e) involved in the lesion similar to the finding of Radaelli et al (2008), recorded in pneumonic lung of veal calves observed at slaughter. *M. bovis*-IHC positive lesions were often centered around bronchiolar structures. These findings provide evidence of bronchogenic nature of *M. bovis* associated supplicative bronchopneumonic lesions (Radaelli et al 2008 and Caswell et al 2010). The presence of focal areas of caseous necrosis, surrounded by inflammatory cells and with a high concentration of *M. bovis* in the inflammatory cells at the margin of the lesions, indicates that the pathological changes may be partly due to the host’s immunoinflammatory response (Thomas et al 1986; Gagea et al 2006; Schott et al 2014 and Rodriguez et al 2015). The persistence of *M. bovis* at the mucosal surface is suggested (Thomas et al 1986; Gagea et al 2006; Radaelli et al 2009 and Rodriguez et al 2015) to be also responsible for the peribronchiolar mononuclear cell infiltration and supplicative bronchiolitis. The similar tissue section when stained without using antibody may not show the immunoreactions (Fig.3d). The immunoreactions in other visceral organs were not observed; hence the histological lesions were not incriminated due to *Mycoplasma bovis*. Mild immunoreactions were noted by Hermeyer et al (2012) in peribronchial macrophages of liver in *Mycoplasma bovis* infected aborted bovine foetus but not in neonatal calves.

Examination of positive immunoreactions revealed granular staining in medium sized necrotic foci with positive reaction in airways gained IHC score ++ in both the cases. The histopathological score for different pathological process within animal’s alongwith IHC score are presented in Table 1.

The scores of histopathological examination (HPE) in immunopositive cases it can be observed that caseonecrotic pneumonia and supplicative bronchopneumonia were most predominant pathological process in *M. bovis*. The other major associated pathological processes included were bronchointerstitial pneumonia and interstitial pneumonia. The statistical correlation could not be calculated in this study due to records of low numbers of positive cases. However significant positive correlation between existence of IHC staining for *Mycoplasma bovis* and the occurrence of pathologic process as recorded in this study reported by Booker et al (2008).

As previously reported (Maeda et al 2003), *M. bovis* immunoreactivity was mainly observed at the periphery of necro-suppurative foci. *M. bovis* immunoreactivity was also
associated with the necrosuppurative exudates plugging bronchiolar lumina and was occasionally observed along the bronchiolar epithelial cell surface, as well as in the cytoplasm of neutrophils involved in the lesions. *M. bovis*-IHC-positive necrosuppurative lesions were often centered around bronchiolar structures. These findings provide evidence of the bronchogenic nature of *M. bovis*-associated necrosuppurative lesions. Although these specific pathological and IHC findings have been widely characterized in previous studies (Adegboye et al., 1995), the exact role of *M. bovis* in the pathogenesis of pulmonary necrosuppurative foci is still poorly understood. It has been postulated that a complex polysaccharide toxin produced by *M. bovis* as well as an excessive host inflammatory response may be involved in the pathogenesis of necrosuppurative lesions (Geary et al., 1981; Rodriguez et al., 1996 and Radaelli et al., 2008). Therefore, *M. bovis* infection should be suspected in bovine lung showing necrosuppurative lesions typical of caseonecrotic and suppurative bronchopneumonia such as abscession (Adegboye et al., 1995) and areas of coagulative necrosis (Khodakaram-Tafti and Lopez, 2004). Since these lesions are also clearly detectable at gross examination they may be considered a useful marker of *M. bovis* infection at postmortem.

The associations of other viral etiologies like IBR, BRSV and PIV3 infection in Mycoplasma positive tissue sections were ruled out by IHC examination using specific individual antibodies.

**Molecular diagnosis of Mycoplasma spp by PCR:** To increase the sensitivity of detection, further polymerase chain reaction assay for Mycoplasma spp was carried out on lung samples of chronic pneumonia cases. PCR amplification was carried out to detect Mycoplasma species following extraction of DNA from lung samples. The PCR protocol employed in this study efficiently amplified the sequences from the positive control sample without any amplification of negative control. Reference strains as well as 2 DNA products were visualized and imaged revealing a product of 280 bp in positive samples (Fig4).

The tested two sample positive by PCR were showed positive to *M. bovis*-immunoreaction staining. The result indicated, PCR as a valuable tool for rapid diagnosis of Mycoplasma infection. The result confirms the two cases of naturally occurring bovine pneumonia due to *Mycoplasma bovis* infection. In this present study the sensitivity and specificity of IHC was in similar to PCR for detection of Mycoplasma spp infection in lung sample. Moreover, immunohistochemical method for Mycoplasma detection is complementary tools in suspected cases with negative bacteriologic culture, in cases when serology is not possible or materials fixed in formalin.

Isolation of *M. bovis* from lungs is not always possible, particularly in chronically affected animals as in the present case, antibiotic treatment; poor lung condition or gross bacterial contamination also inhibits isolation. Here, immunohistochemical techniques preferably using monoclonal antibodies may be valuable to visualize the Mycoplasma antigens in the affected tissue (Adegboye et al., 1995). Immunohistochemical demonstration of *M. bovis* antigen within tissues is a sensitive and specific means of determining the involvement of *M. bovis* in observed pathology (Adegboye et al., 1995; Rodriguez et al., 1996; Haines et al., 2001; Clark 2002; Shahriar et al., 2002; Maeda et al., 2003; Khodakaram-Tafti and Lopez, 2004 and Gagea et al., 2006). Advantages of IHC are that it performs well using formalin fixed, paraffin embedded tissues, and can be performed retrospectively, especially when other findings suggest a *M. bovis* infection but culture is negative. An additional advantage of IHC is that it reveals the location of *M. bovis* within lesions. The PCR has been most commonly adopted for diagnosis of Mycoplasma bovis because of its high sensitivity and specificity. The results of this study are in agreements with several previous studies that reported on the usefulness of PCR and IHC and advocated its use for the diagnosis of Mycoplasma infection on postmortem sample (Pinnow et al., 2001; Bashiruddin et al., 2005; Hotckiss et al., 2010 and Colin et al., 2014).

**CONCLUSION**

The study demonstrated a record of *Mycoplasma bovis* infection on screening of 87 fatal pneumonia cases in bovine. The study confers that *M. bovis* infection may develop into a severe necrosuppurative bronchopneumonia when associated with high numbers of intralesional organism and into mild catarhal bronchointerstitial pneumonia when associated with low numbers of organism. The sensitivity of PCR and immunohistochemistry were equally useful method and can be advocated for the diagnosis of Mycoplasma infection.

**REFERENCES**


