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Pathological changes and immunohistochemical characterization of lung lesions in small ruminants naturally infected with *Mycoplasma agalactiae*

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Abstract
Respiratory ailments caused by mycoplasma organisms are considered as important diseases of small ruminants causing a serious economic impact. The present work describes the pathological changes in lungs of sheep and goat naturally infected with *Mycoplasma agalactiae* organisms. Pneumonic lungs from sheep and goat (n=30) were collected from slaughter houses and *M. agalactiae* was isolated from 8 cases. The histopathological changes in the infected lungs were hyperplasia of bronchial epithelium, hyperplasia of BALT (bronchus associated lymphoid tissue) and infiltration of mononuclear cells around the airways and blood vessels, suggestive of broncho-interstitial pneumonia. The presence of the organism in the lesions was also demonstrated by immunohistochemistry using polyclonal hyperimmune serum raised against *M. agalactiae*.

Keywords: Mycoplasma agalactiae, sheep, goat, pneumonia, immunohistochemistry

Introduction
Mycoplasma agalactiae is considered as the main causative agent of contagious agalactia (CA) in sheep and goats [1]. CA is usually manifested as mastitis in lactating females. In males and non lactating females it causes arthritis, Keratoconjunctivitis and pneumonia in different age groups [2, 3]. *M. agalactiae* causes chronic and persistent infections in small ruminants and the infected animals continue to shed the bacteria for several months, sometimes for several years [4]. The presence of asymptomatic carriers in a herd appears to be a serious health risk. Such animals easily escape disease control and eradication measures, and are capable of flaring up frequent CA outbreaks under stress conditions [5]. Despite causing significant economic losses, mechanisms of infection and persistence of *M. agalactiae* are largely unknown.
Perusal through the available literature revealed very few reports describing the lung lesions in natural cases of *M. agalactiae* in small ruminants. In addition, immunohistochemical detection of the organism in lung lesions has been demonstrated only in a few experimental cases [6, 7]. In the present study, mycoplasma organisms were isolated from pneumonic lungs of sheep and goats using culture, and *M. agalactiae* was identified by PCR. The histopathological changes in lungs of sheep and goat naturally infected with *M. agalactiae* have been described. The presence of the organism in the lesions was also demonstrated using immunohistochemistry using hyperimmune *Mycoplasma agalactiae* serum.

Materials and method
Collection of Samples
Lungs from slaughtered sheep and goat, which showed gross lesions suggestive of pneumonia were collected from the local slaughter house. Two pieces of lung tissue were collected from each case. One piece was placed in the Mycoplasma broth medium for isolation of causative organism and the other half was placed in 10 per cent neutral buffered formalin for histopathological studies. Thin sections of 4 µm thickness were obtained from the collected samples from lungs and were stained by haematoxylin and eosin method.

Isolation and Identification of Mycoplasma
The lung tissues of approximately one cubic mm in size were incubated aerobically for 4 hours in pleuropneumonia like organism (PPLO) broth. The mycoplasma broth was further

incubated for 3-4 days after removal of lung pieces. The turbid cultures were further streaked on PPLO agar media the agar plates were incubated at 37◦C in a candle lit jar for 10% CO_2 tension for 6–8 days. Mycoplasmas were isolated on the basis of appearance of "nipple" shaped colonies embedded in the media. Identification of *M. agalactiae* was done using PCR as per Tola *et al*. (1996) [8].

Detection of Bacterial Antigen by Immunohistochemistry

Lung tissue samples from mycoplasma infected cases were selected for immunohistochemistry (IHC). Thin sections of 4 µm thickness were obtained on 3-Aminopropyl – triethoxysilane coated slides. The sections were deparaffinized and rehydrated. Antigen retrieval was achieved by immersing the slides in citrate buffer followed by microwaving for 5 min. The slides were then allowed to cool for 20 min, rinsed thoroughly in PBS and placed in PBS wash bath for fifteen minutes. Endogenous peroxidase was blocked using 3% Hydrogen Peroxide in methanol at room temperature for 15-20 minutes. Blocking of non-specific sites were done with BG-PBST for one hour at RT. Sections were incubated overnight at 4^0C in a humidified chamber with polyclonal rabbit hyperimmune serum raised against *M. agalactiae*, followed by incubation with anti rabbit IgG conjugated with HRPO at the dilution of 1:400 at 37 ^{0}C for one hour. Finally, freshly prepared 3,3-diamine benzidine tetra hydrochloride (DAB) substrate with 3% H_2O_2 was poured to cover the sections and incubated for 5-10 minutes or until the desired color developed at room temperature. After washing the sections with PBS, the sections were counterstained with Harris haematoxylin for two minutes. The stained sections were washed in distilled water, dehydrated, cleared in xylene, cover slipped with DPX and examined under the microscope.

Results

The gross lesions observed in the pneumonic lungs collected from slaughter houses included areas of reddish gray purple and consolidation involving different lobes. Part of the pneumonic lung samples collected from slaughter houses for isolation of mycoplasmas were subjected to routine histopathology. The histopathological changes in the 8 lung samples, from which *M. agalactiae* was isolated and identified by culture and PCR, were suggestive of a bronchointerstitial pneumonia characterized by hyperplasia of bronchial epithelium, hyperplasia of BALT (bronchus associated lymphoid tissue) and infiltration of mononuclear cells around the airways and blood vessels (Fig. 1, 2). The interstitium was thickened due to infiltration of neutrophils, macrophages and lymphocytes and proliferation of fibrous tissue.

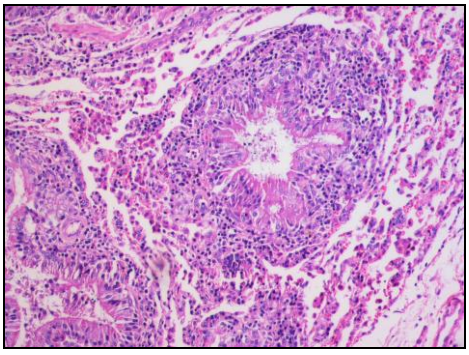

Fig 1: Lung showing interstitial pneumonia with accumulation of inflammatory cells around the bronchiole. H&E x 100

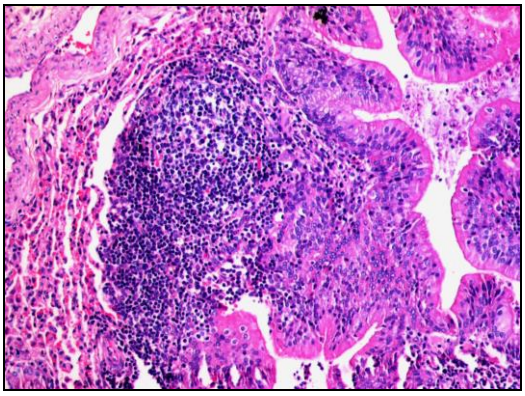

Fig 2: Lung showing hyperplasia of bronchiolar epithelium and BALT, also note inflammatory exudate in the bronchiolar lumen. H&E x 100

Immunohistochemical staining of lung sections with *M. agalactiae* hyperimmune serum revealed positive staining on the mucosal surface of the epithelium and in the cytoplasm of the epithelium of the bronchi and bronchioles (Fig. 3). Mycoplasma antigens were also present in the lumen of the airways and alveoli, mainly inside the cytoplasm of neutrophils and macrophages (Fig. 4).

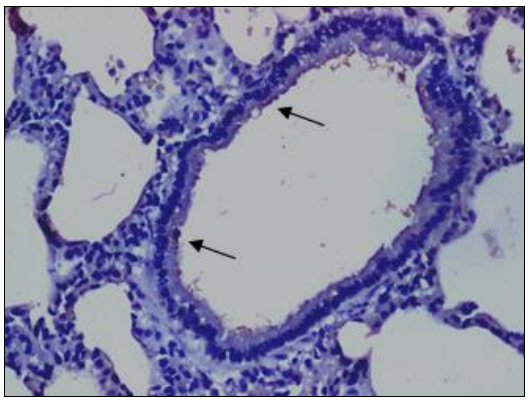

Fig 3: Lung showing immunostained *M. agalactiae* antigen in the mucosal surface, cytoplasm of bronchiolar epithelium (arrow). IP x DAB x 200

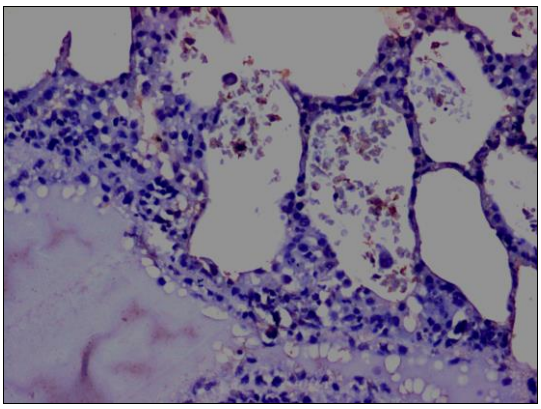

Fig 4: Lung showing positive immunostaining in the exfoliated epithelium and inflammatory cells. IP x DAB x 200

Discussion

In small ruminants, mycoplasmas are considered as an important class of bacteria responsible for respiratory infections which can be inapparent or can cause a mild, acute or chronic disease [9]. In the present study, the gross lesions observed in the pneumonic lungs collected from slaughter

houses, from which *M. agalactiae* was later isolated, were areas of discoloration and consolidation involving different lobes and these were in accordance with the observations of previous workers in lungs infected with mycoplasma [10, 11, 12, 13]. The histopathological changes in the 8 lungs from which *M. agalactiae* was isolated and identified by culture and PCR, were suggestive of bronchointerstitial pneumonia characterized by thickening of interstitium due to infiltration of neutrophils, macrophages and lymphocytes and proliferation of fibrous tissue. Hyperplasia of bronchial epithelium, hyperplasia of BALT and infiltration of mononuclear cells around the airways and blood vessels were also noticed. These findings were similar to the observations of previous workers in mycoplasmal pneumonia [10, 11, 14, 15, 16].

The histopathological changes in lungs reported in natural cases of CA (contagious agalactiae) are cuffing interstitial pneumonia, and is characteristic of mycoplasmal infections[17]. The common feature of mycoplasmal pneumonia is the increase of mononuclear cells in the lung, which accumulate in peribronchiolar and perivascular areas. The aggregation of inflammatory cells into well-organized lymphoid follicles is a characteristic feature of chronic mycoplasmal lesions, and is described in both natural and experimental infections [18, 7]. The persistence of mycoplasmal organisms at the mucosal surface has been linked to the formation of lymphoid deposits around airways [18]. These accumulated cells are likely to synthesize antibody, indicating the significant role of cellular immunity [19, 18, 7]. In addition, cell-mediated immunity effected by T lymphocytes has two major effects, namely increasing the phagocytic and cytotoxic activities of macrophages, and initiating the chronic inflammatory response. Synthesis of interleukin I and TNF alpha by activated macrophages may also have profound effects on the immune system.

In the present study, immunohistochemical staining with *M. agalactiae* hyperimmune serum revealed positive staining on the mucosal surface of the epithelium and in the cytoplasm of the epithelium of the bronchi and bronchioles. This proves the persistence of mycoplasmal organisms at the mucosal surface leading to a chronic infection [18]. Similar immunostaining has been reported for *M. ovipneumoniae* in pneumonic sheep lungs [20, 21]. Mycoplasma antigens were also present in the lumen of the airways and alveoli, mainly inside the cytoplasm of neutrophils and macrophages reemphasizing the role of cell mediated immunity against mycoplasmal infections. Cell invasion is believed to play a major role in the systemic spreading of many pathogens, including mycoplasmas. The capability of *M. agalactiae* to enter host cells might be the strategy that it employs at a population level to ward off the host immune response and antibiotic action, and to disseminate to new and safer niches to later egress and once again proliferate upon the return of favorable conditions to cause persistent chronic infections [21]. Presence of intracellular mycoplasmas has been clearly substantiated using immunohistochemistry in the present study. Internalized *M. agalactiae* could survive and exit the cells in a viable state to repopulate the extracellular environment after complete removal of extracellular bacteria with antibiotics [22]. This study is the first report of the isolation and identification of mycoplasmas from lungs of small ruminants collected from slaughter houses in Bangalore, India. This isolation is significant because mycoplasmas are one of the major causative agents of small ruminant diseases and have gone unnoticed or attributed to other causes due to the difficulties in their diagnosis. In the Mediterranean region CA alone is estimated to cost at least US$ 30 million annually mainly as the result of milk production losses but mortality and poor growth in the young may also be significant [1]. Respiratory diseases are considered as the most important diseases of small ruminants causing a serious economic impact [23, 24, 25] and mycoplasmas are considered an important cause for respiratory infections in small ruminants.

The existence of mycoplasma organisms in the lungs as demonstrated by IHC, confirms their importance in the disease process. *M. agalactiae* was the major isolate in the present study and is similar to the situation in Europe, where the majority of isolates from cases of CA in sheep and goats were *M. agalactiae* [26]. The emergence of CA in a non-infected herd is almost always linked to the introduction of carriers of CA or to contact with infected herds. There is rapid spread of clinical problems in the herd coinciding with the period of parturitions and the start of lactation [27]. Thus, many infected pneumonic animals may pass unnoticed and may act as a permanent source of infection. The present study emphasizes the need for an established and systemized diagnostic process to identify the carrier animals.

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