Immunohistochemical detection of *Mycoplasma mycoides mycoides* small colony type in lungs of slaughtered cattle at Morogoro slaughterhouse, Tanzania

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SUMMARY

Diagnosis of contagious bovine pleuropneumonia (CBPP) in carrier animals remains to be a challenge in developing countries partly because of lack of diagnostic tools that can detect asymptomatic animals. As a result many apparently healthy animals pose a potential impending epidemic in periods of stress. We examined condemned lungs with CBPP-like lesions from apparently healthy slaughtered animals to confirm presence of the disease in these animals. A total of 13 lungs collected at abattoir were examined immunohistochemically using 3H12 and 6E3 monoclonal antibodies against *Mycoplasma mycoides mycoides* (MmmSC), the causative agent of CBPP. Both 3H12 and 6E3 antibodies detected the MmmSC antigens in all the 13 lungs in bronchial, bronchiolar and alveolar epithelia, bronchial glands, bronchial cartilage, and in alveoli. We conclude that CBPP is still present in various places of Tanzania, posing a threat to livestock production and that immunohistochemistry can be an efficient diagnostic method to confirm presence of the disease in asymptomatic healthy animals passed for slaughter.
Keywords: CBPP, cattle, monoclonal antibody, lung parenchyma, MmmSC antigens, immunohistochemistry

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP), caused by *Mycoplasma mycoides mycoides* small colony (MmmSC) type, is an acute, sub acute or chronic infectious disease of cattle and water buffaloes. In its acute phase, CBPP is characterized by severe exudative inflammation of the lungs, serofibrinous pleuritis and heavy mortalities. The lung lesions may undergo sequestration in which case the MmmSC is contained in circumscribed lesions (sequesters) and the infection becomes latent as the disease enters a chronic phase. Chronically infected animals become poor milk and meat producers and are asymptomatic potential carriers of new infection (Mbulu *et al.*, 2004). The new infection occurs under stressful conditions such as drought and long distance trekking that cause sequesters to rupture and release the MmmSC. This has led to extension of endemic, re-emerging or epidemic CBPP infections throughout the pastoral herds of western, central and eastern Africa (Windsor 2000; Miltiadou *et al.*, 2009). Indeed, uncontrolled cattle movement in search of water and pasture contributes significantly to the eruption and spread of the disease from an apparent healthy herd (Ocaido *et al.*, 2009).

While effective vaccination is the viable control policy at present in most African countries, no single vaccine has proven efficient in eradicating CBPP. Vaccines like combined Rinderpest and CBPP vaccine (Bisec vaccine), T1-SR vaccine, and T1-44 that have once been used or are

currently in use have proven ineffective in various countries (Melewas 1999; Abdo *et al.*, 2000; Thiaucourt *et al.*, 2000; Mbulu *et al.*, 2004; Kitalyi 2005). Further, vaccination programs have been difficult to implement in many pastoral communities in Africa partly because of wide infected area, limited resources, failure of herdsmen to present the whole herd for vaccination or repeat the vaccination as required, and their refusal to continue vaccination following vaccination reactions (Melewas 1999; Kitalyi 2005). The highly challenged vaccination programs have resulted in spots of unvaccinated animals and more virulent field strains that have been potential sources of outbreaks now and again. Unfortunately, these outbreaks are loosely handled leading to more wide spread of infections from apparently healthy animals.

Efficient control of CBPP not only requires more efficient vaccine strains, but also reliable diagnostic strategies (Mbulu *et al.*, 2004; Pilo *et al.*, 2007; Bischof *et al.*, 2009). In Africa, diagnosis of apparently healthy animals plays an important role to the control of the disease. The diagnosis could be followed by effective vaccination in quarantined area and surveillance. Unfortunately diagnostic tests that permit detection of asymptomatic carriers as well as those applicable under field conditions are lacking. The World Organization for Animal Health (OIE) recommends complement fixation test as an international diagnostic test for CBPP. Enzyme linked immunosorbent assay (ELISA) is another commonly used diagnostic method. However, both tests are expensive, need well trained personnel to perform, and are unsuitable for field application and mass screening in poor societies of Africa. In this respect, meat inspection at abattoirs to discover lung lesions is probably a promising diagnostic approach to asymptomatic carriers. Identified lungs with CBPP lesions can further be studied grossly, histologically or microbiologically for confirmatory diagnosis. In this study, we used immunohistochemistry to confirm presence of MmmSC in apparently healthy animals slaughtered at abattoir.

MATERIALS AND METHODS

Study area

The study was conducted in Morogoro (Tanzania) urban abattoir between November 2011 and April 2012. This abattoir receives animals from various areas as far as a diameter of 200 km away. The study involved visiting the abattoir to collect condemned lungs that had lesions typical of CBPP i.e. consolidated, fibrinous, edematous, and with enlarged interlobular septa. Owing to the scarcity of the cases, the visit followed a phone call from the meat inspector on duty after identifying the lungs.

Gross examination of samples

Collected lungs were transported to the laboratory (Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro). Thorough gross examination was done after which, some pieces of lungs were taken and fixed in 10% neutral buffered formalin (NBF) for at least 24 hours.

Immunohistochemistry

Chrome-gelatin subbing

Subbing of microscopic slides was done to prevent detachment of tissue sections from the slide during immunohistochemistry. Chrome-gelatin subbing solution was made by dissolving 10 g of gelatin and 1 g of $CrK(SO_4)_2.12H_2O$ in 1 liter of warm non-boiling distilled water. After stirring until

dissolved, a thymol crystal was added. The solution was allowed to cool before further dissolving 0.188 g of $CrK(SO_4)_2.12H2O$ in the solution. This subbing solution was used on the same day to avoid infection with micro-organisms. Microscopic slides were placed in racks and soaked in soap solution (1 hr) then rinsed in deionized water that was changed several times to remove all the soap. The slides were then dipped into the subbing solution, drained onto a paper towel and allowed to air dry for 1 hr. A second dipping was done followed by draining and loosely covering the slides with plastic wrap or bench paper. When thoroughly dry, the slides were stored in slide boxes until use.

Preparations of tissue sections

The formalin fixed tissues were processed and embedded in paraffin in a routine manner. After obtaining tissue blocks, sectioning was done by rotary microtome to obtain 4 microns tissue sections. The thin tissue sections were applied to the subbed microscopic slides, deparaffinized in xylene (3 changes, 5 min each), hydrated through graded alcohol (100% twice then 95% ethanol twice; 10 min each), and washed in deionized water for 1 min with stirring. Excess liquid was aspirated from slides.

Heat treatment antigen unmasking

The microscopic slides with tissue sections were placed in a container and covered with 10 mM sodium citrate buffer (pH 6.0). They were heated at 95°C for 5 min. The container was topped off with fresh buffer and heated again (95°C, 5 min). The slides were allowed to cool while in the buffer (20 min) and subsequently washed in deionized water (3 times, 2 min each). Any excess liquid was aspirated from slides.

Quenching endogenous peroxidase activity

To quench the endogenous peroxidase activity, tissue sections on slides were incubated for 30 min in 3% H_2O_2 in deionized water. The slides were then washed (3 times, 5 min each) in phosphate-buffered saline (PBS) (0.01 M Na_2PO_4, 0.01 M NaH_2PO_4, 0.9% NaCl, pH 7.3).

Immunoperoxidase staining using Horseradish peroxidase-Streptavidin system

Immunostaining was done by Horseradish peroxidase (HRP)-Streptavidin system using SPlink HRP detection bulk kit (GBI Labs, Mukilteo, WA, USA; Cat No D01-60) in accordance with manufacturer's recommendations. Briefly, the tissue sections were completely covered with pre-blocking solution, incubated for 10 min, and the solution blotted off without rinsing. Monoclonal MmmSC antibodies 3H12 (mouse ascites fluid) and 6E3 (caprylic acid purified tissue culture fluid (both offered in kind by Dr Cathy Brooks of Agri-Food Biosciences Institute, Belfast, UK) diluted in 2% normal blocking goat serum in PBS were applied to cover the tissue sections completely and incubated in moist chamber for 1 hr. After rinsing with PBS (2 min, 3 times), a biotinylated secondary antibody broad spectrum was applied to cover the tissue sections completely and incubated for 10 min in moist chamber. The sections were then rinsed with PBS (2 min, 3 times), completely covered with HRP-Streptavidin, incubated in moist chamber for 10 min, and rinsed again with PBS (2 min, 3 times). Pre-mixed 3,3-diaminobenzidine plus (DAB+) chromogen (adding 1 drop of DAB chromogen concentrate in 1 ml of DAB substrate buffer and mixing well; GBI Labs,

Mukilteo, WA, USA; Cat No C09-12) was applied to completely cover the tissue sections, incubated for up to 5 min, and gently rinsed under tap water (1-2 min) when appropriate color developed. The sections were then counterstained with Harris's hematoxylin (10-20 sec), rinsed thoroughly under tap water (1-2 min), put in PBS until they showed blue color (30-60 sec) and rinsed well in deionized water. The sections were then dehydrated in graded ethanol (95% twice then 100% twice; 2 min each) and cleared in xylene (2 min, twice). Finally, the slides were mounted (DPX), observed under light microscope (Olympus BX41, Japan) and photograph taken by DP21 camera (Olympus, Japan).

Control samples

Both normal and pathological cattle lung tissues were also processed for immunohistochemical staining as controls. They were divided and treated in three ways: those receiving primary monoclonal 3H12 or 6E3 MmmSC antibodies but not biotinylated secondary antibody, those receiving 2% normal blocking goat serum in PBS without primary monoclonal 3H12 or 6E3 MmmSC antibodies but receiving biotinylated secondary antibody, and those receiving neither primary monoclonal 3H12 or 6E3 MmmSC antibodies nor biotinylated secondary antibody.

RESULTS

Immunohistochemical staining detects targeted tissue antigens using specific antibodies. In this study, MmmSC antigens were detected in various locations in the lung tissues by their specific 3H12 and 6E3 monoclonal antibodies. The locations included lung parenchyma, bronchi, bronchioles and blood vessels.

As depicted in Figure 1, the whole affected lung parenchyma stained positive for MmmSC antigens as evidenced by brownish discoloration/precipitation, indicating the specific reaction of 3H12 or 6E3 monoclonal antibodies to MmmSC antigens. The antigens were located in the alveoli as well as the interalveolar septa. On higher magnification, the staining appears to be intracytoplasmic in macrophages and alveolar epithelial cells (Figure 2). It is clear in both Figures 1 and 2 that the antigenic staining was more on severely affected areas than in moderately affected ones where the lung architecture is well appreciated.

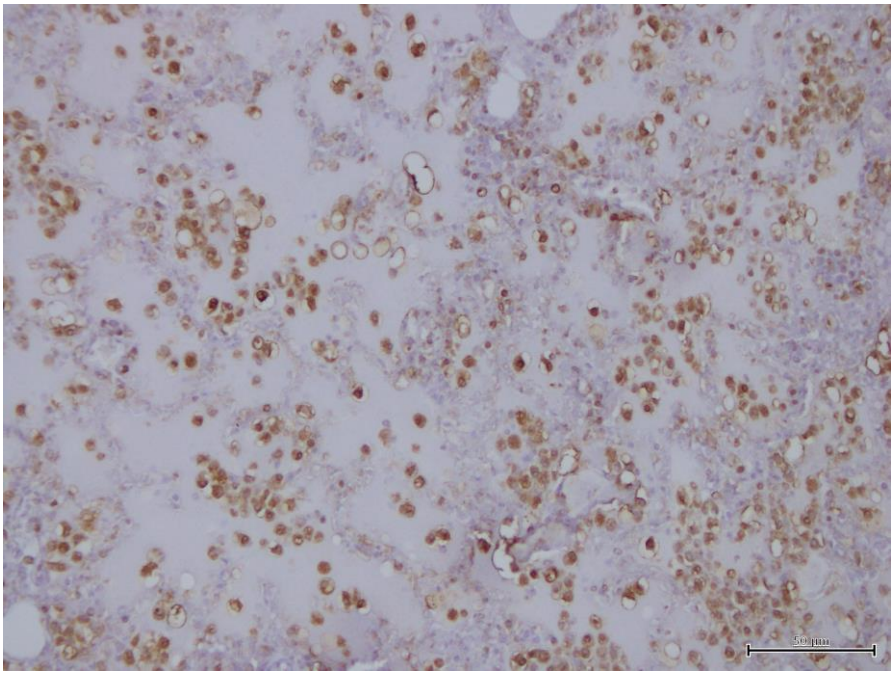

Figure 1. Histological section of a cattle lung with CBPP. The section was stained with monoclonal antibody 3H12 against MmmSC antigens and detected by 3,3-diaminobenzidine plus (DAB+). The brownish discoloration throughout the section indicates positive reaction for MmmSC antigens. x200.

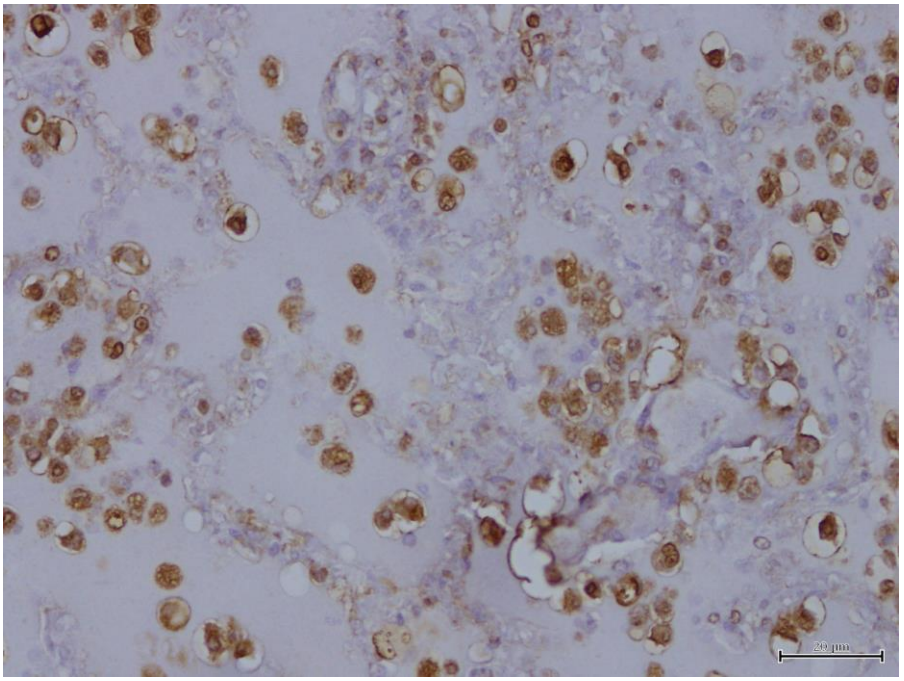

Figure 2. Histological section of cattle lung with CBPP stained with monoclonal antibody 3H12 showing brownish discoloration due to MmmSC antigens. Antigens in macrophages and alveolar epithelial cells are manly intracytoplasmic. x400

The MmmSC antigens were heavy in expanded interalveolar area even when the alveoli were still appreciated (Figure 3). In these areas all the structures are staining positive indicating presence of MmmSC antigens throughout the interstitium.

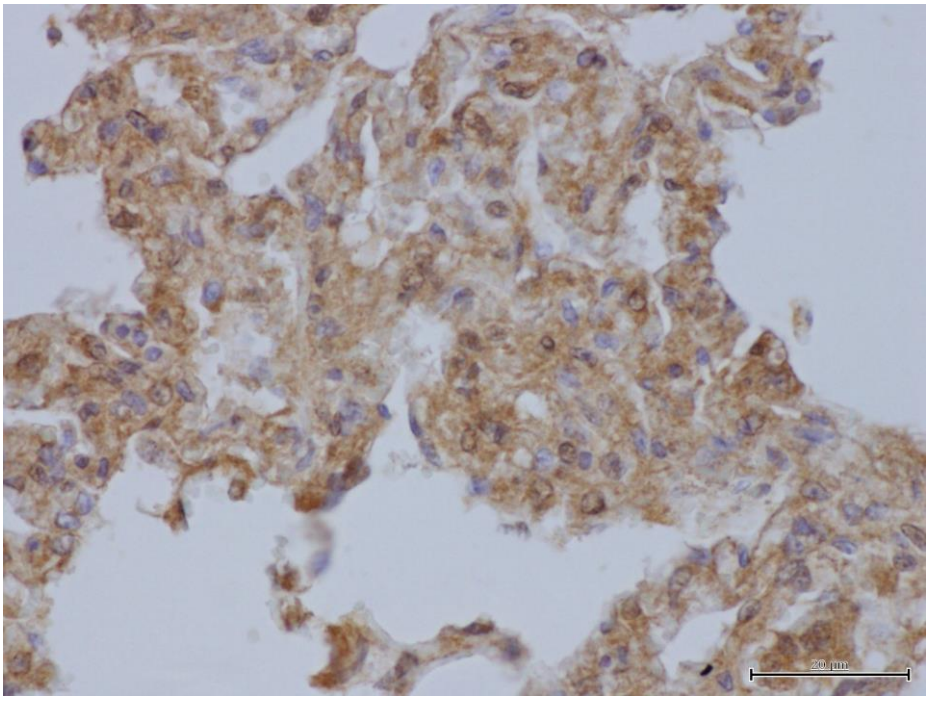

Figure 3. Histological section of cattle lung stained with monoclonal antibody 6E3 to detect MmmSC antigens. There was heavy brownish discoloration of all structures in expanded interalveolar area. x600

The monoclonal antibodies were also able to detect MmmSC antigens in and around blood vessels, bronchi and bronchioles. Figures 4 and 5 show clearly that in addition to picking MmmSC antigens in the lung parenchyma, the applied monoclonal antibodies also demonstrated presence of MmmSC antigens in and around blood vessels, bronchi, and bronchioles. It is evident from these figures that the MmmSC antigens were present in the epithelium as well as in the lumen. A large magnification of a bronchus clearly indicates the presence of MmmSC antigens in the epithelium (Figure 6).

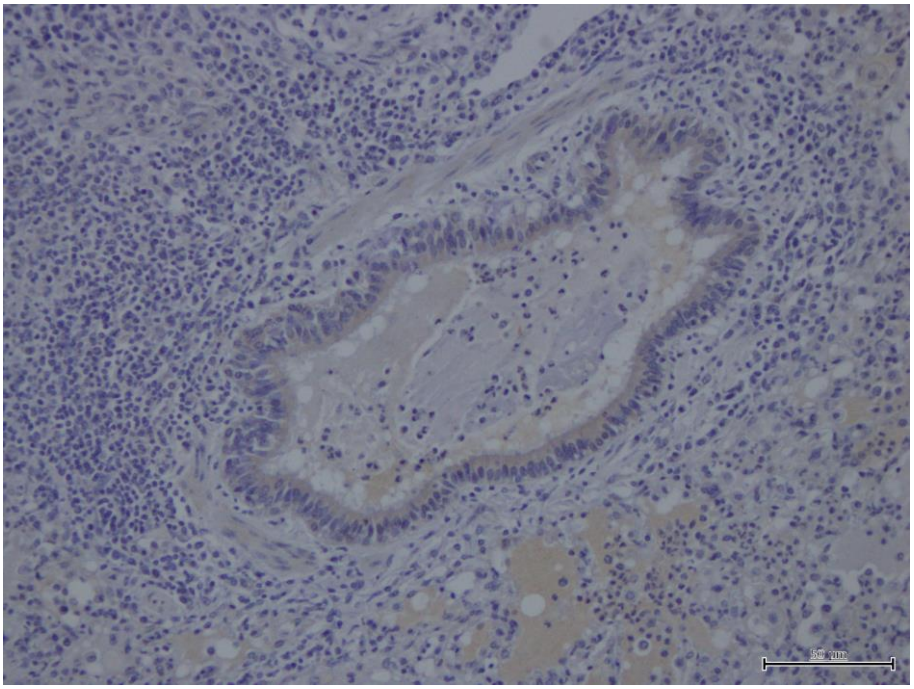

Figure 4. Histological section of cattle lung stained with monoclonal antibody 6E3 to detect MmmSC antigens. There was heavy peribronchiolar lymphoplasmacytic infiltration and presence of MmmSC antigens in and around the bronchiole as well as within bronchiolar epithelium. x200

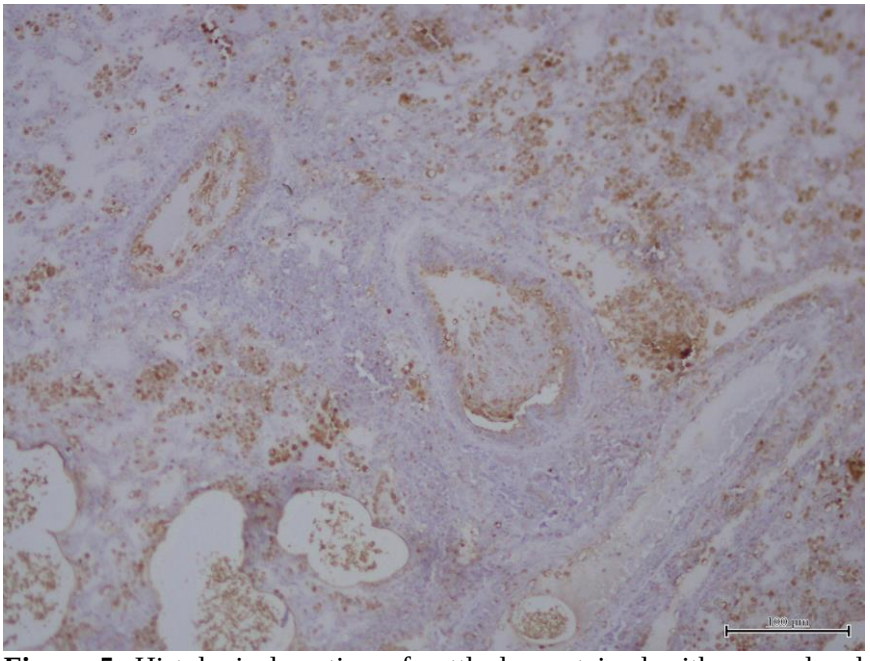

Figure 5. Histological section of cattle lung stained with monoclonal antibody 3H12 to detect MmmSC antigens. The MmmSC antigens are located in the lung parenchyma as well as in and around blood vessels and bronchi. x100

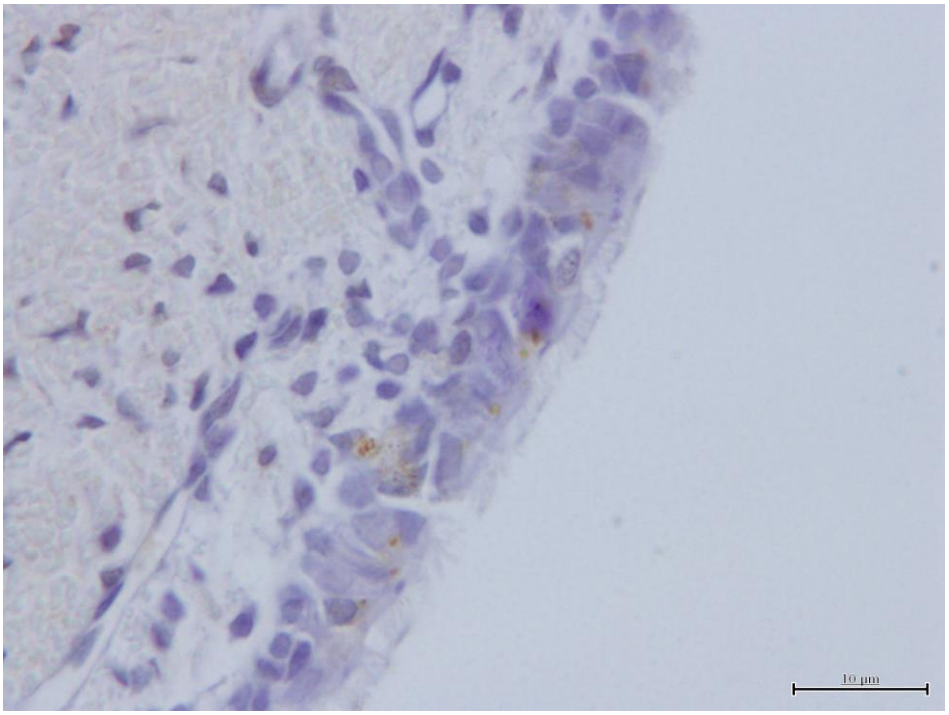

Figure 6. Histological section of part of a bronchus of cattle stained with monoclonal antibody 3H12 demonstrating presence of MmmSC antigens in bronchial epithelium. x1000

A thorough review of the bronchus indicated that the MmmSC antigens were also demonstrated in sero-mucous glands in the submucosa (Figure 7) and in the cartilage framework (Figure 8). The MmmSC antigens were intracytoplasmic in all cases.

Immunohistochemical staining of normal lungs using the 3H12 and 6E3 antibodies (data not shown) or of affected lungs without either of the two MmmSC antigens (Figure 9) did not show any brownish discoloration/precipitation alluding to the lack of antigen-antibody reaction.

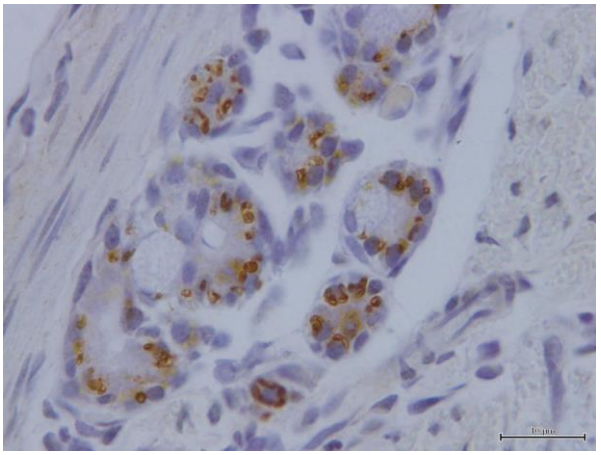

Figure 7. Histological section of part of a bronchus of cattle stained with monoclonal antibody 3H12 indicating presence of MmmSC antigens in sero-mucous glands in the bronchial submucosa. x1000

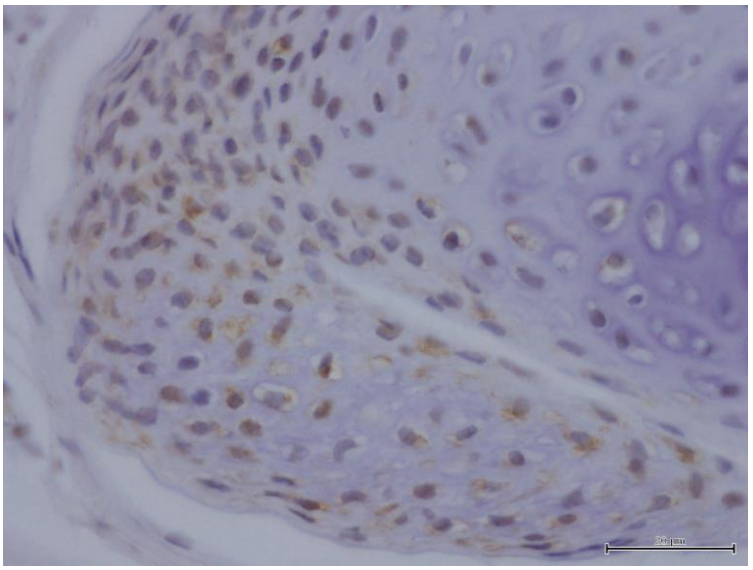

Figure 8. Histological section of part of a bronchus of cattle stained with monoclonal antibody 3H12 indicating presence of MmmSC antigens in the bronchial cartilage framework. x600

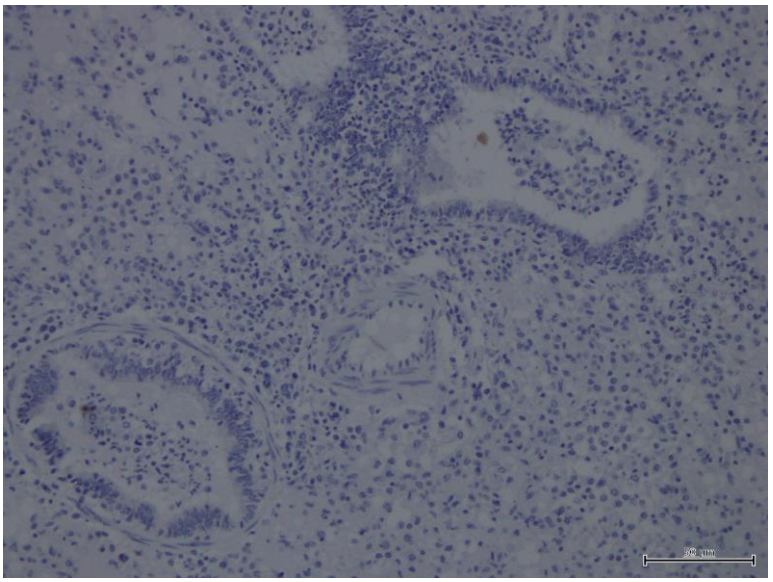

Figure 9. Histological section of cattle lung with CBPP. The staining excluded both 3H12 and 6E3 monoclonal antibodies for detection of MmmSC antigens but included the secondary antibody and detection step by DAB+. There was not brownish discoloration to indicate positive reaction for MmmSC antigens. x200

DISCUSSION

Diagnosis of CBPP in endemic areas in Africa and Asia is challenged by lack of capacity to diagnose carrier asymptomatic animals that pose potential impending epidemics. Several such epidemics occurred in sub-Saharan Africa, including

Tanzania in late 1990 (Bölske *et al.,* 1995; Melewas 1999; Kapaga *et al.,* 2005). The control measures instituted to these epidemics included limited cattle movement and vaccination. Unfortunately, due to inefficient vaccine, wide infected area, limited resources, failure of herdsmen to present the whole herd for vaccination or repeat the vaccination as required, and their refusal to continue vaccination following vaccination reactions (Melewas 1999; Abdo *et al.,* 2000; Thiaucourt *et al.,* 2000; Mbulu *et al.,* 2004; Kitalyi 2005) many animals were not vaccinated. This created spots of apparently healthy infected carrier animals that kept on spreading the disease. Worse, these animals cannot be diagnosed and thus endemic, re-emerging or epidemic CBPP infections extend throughout the pastoral herds of much of western, central and eastern Africa (Windsor 2000; Miltiadou *et al.,* 2009).

One of the major factors to the continual spread of CBPP is the incapability to diagnose carrier asymptomatic animals. Thus developing diagnostics that will identify infected animals even at the subclinical and carrier states is a pressing priority. Currently diagnosis is possible at the abattoir during meat inspection since the pathological lesions of CBPP are distinctive (Ferronha *et al.,* 1990; Di Francesco *et al.,* 1998). Indeed, the World Organisation for Animal Health, OIE, approves abattoir surveillance of the disease by examining lungs with CBPP-like lesions as a practical method for disease monitoring (OIE 2008). In this study we have demonstrated the use of immunohistochemistry to detect MmmSC antigens, the causative agent of CBPP, in condemned abattoir lungs with CBPP-like lesions. All the 13 collected lung samples were positive for MmmSC antigens when tested with either 3H12 or 6E3 monoclonal antibodies. The MmmSC antigens were detected in various locations in the lung parenchyma, bronchi, bronchioles and the blood vessels. The application of immunohistochemistry to detect MmmSC has been proven to be a robust assay in the diagnosis of CBPP by other scholars, particularly where the causative organism is not recoverable (e.g. following long transport distances), where the animal has died of acute disease, or where serology cannot be performed or is inconclusive (Ferronha *et al.,* 1990; Scanziani *et al.,* 1997).

Contrary to immunohistochemistry which detected MmmSC antigens in all the 13 samples collected, only 2 (15.4%) cultures grew Mycoplasma organisms (data not shown). This indicates the reliability of immunohistochemistry in confirming CBPP in apparently healthy carrier animals even when isolation or other methods are ineffective. Other researchers have also noted more or less similar results. For instance, in an examination of 11 CBPP affected lungs from Portuguese cattle, immunohistochemistry detected all, while polymerase chain reaction (PCR) and culture detected 5 (45.5%) and 4 (36.4%) cases respectively (Ayling *et al.,* 1998). In addition, Cetinkaya *et al.* (2003) cultured 62 abattoir lung samples from Turkish cattle with lesions suggestive of CBPP but only 3 (4.8%) samples grew Mycoplasma species. One reason for this difference is that immunohistochemistry is capable of detecting Mycoplasma even in decomposing tissue from which isolation may prove difficult partly due to non-viable microbes (Adegboye *et al.,* 1995).

Demonstrated in this study is also the ability of immunohistochemistry to detect the MmmSC antigens at specific sites in the lung. A thorough study on this staining may lead to establishment of the exact pathogenesis of CBPP which will subsequently assist in the control of the disease. This is a major attribute of

immunohistochemistry compared to other methods like complement fixation test, ELISA and PCR that merely confirm the presence or absence of MmmSC and do not provide a perfect match to the typical CBPP pathological findings (Bölske *et al.*, 1995; Cetinkaya *et al.*, 2003; Bashiruddin *et al.*, 2005). Indeed, immunohistochemistry results depicted in this study were a complete match to gross diagnosis of CBPP in all the condemned lungs.

Availability of MmmSC antibodies is scarce. Prior to conducting this study we searched from several producers (companies) of biologicals but we found none. Fortunately we got 6E3 and 3H12 monoclonal antibodies in kind from Dr Cathy Brooks of Agri-Food Biosciences Institute, Belfast, UK. Brooks *et al.*, (2009) have used these antibodies to detect MmmSC antigens by sandwich ELISA that provides a suitable assay for screening large numbers of samples for CBPP. There is therefore no doubt that the positive staining observed in this study was specific to MmmSC strains. In reality, when either of the monoclonal antibodies was omitted, the samples stained negative. This is the first study demonstrating immunohistochemically the presence of MmmSC antigens in lung tissue using the 6E3 and 3H12 monoclonal antibodies.

Compared to other diagnostic methods and the lack of diagnostics that can detect carrier animals, abattoir screening of animals remains to be the best option in monitoring CBPP in developing countries where slaughter and compensation is impossible. Abattoir examination of lungs for CBPP diagnosis is cheaper and can easily be handled by meat inspectors. However, to confirm the disease, techniques employing immunohistochemistry principle or improved version of immunohistochemistry that is user friendly to stakeholders will be the best answer to confirmatory diagnosis of CBPP in Africa.

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