



Immunohistochemical Evidence of Multiple Viral and Bacterial Associations in Caprine Pneumonia in Nigeria: Implications for Vaccines

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Abstract

Caprine pneumonia is a major cause of economic loss and the conventional vaccines are not optimal in protecting goats. A better understanding of the associations of respiratory pathogens may help improve our knowledge for vaccination to effectively control caprine pneumonia. One hundred and fifty goats (140 pneumonic and 10 normal) were examined for various lung pathologies using standard gross and histologic techniques. Antigens of parainfluenza 3 virus (PI3V), respiratory syncytial virus (RSV) and peste des petits ruminants virus (PPRV) and bacterial antigens of *Mannheimia haemolytica* (*M. haemolytica*) and *Pasteurella multocida* (*P. multocida*) were demonstrated immunohistochemically in the lungs. The data of goats positive and negative for the viral and bacterial antigens were analysed using descriptive statistics.

Viral antigens were detected in 113 (81%) of the pneumonic lungs (100 as single, 11 dual and 2 triple). Bacterial antigens were detected in 120 (86%), *M. haemolytica* in 47 (34%),

P. multocida in 59 (42%) and combined bacterial antigens in 14 (10%) of the pneumonic lungs. Multiple agents were detected in 108/140 positive cases; virus-bacterium association was observed in 106/108. PPRV antigens alone were observed in 15 cases. PPRV coexisted most frequently with *M. haemolytica* (n=20), *P. multocida* (n=13), PI3V with *P. multocida* (n=18), and RSV with *M. haemolytica* (n=9). The lesions corresponded to cranioventral (n=45), diffuse (n=75), and lobar consolidations (n=20) manifested as fibrinous bronchopneumonia (n=22), suppurative bronchopneumonia (n=20), bronchointerstitial pneumonia (n=61), interstitial pneumonia (n=25) and bronchiolitis (n=12). Thus, multiple infections are involved in pneumonia, hence we must consider combined vaccination strategies incorporating multiple antigens for adequate control of caprine pneumonia.

Keywords: Goat pneumonia, pathogens, *Pasteurella*, *Mannheimia*, PI3V, PPRV, RSV

Introduction

Pulmonary lesions in livestock arise due to infection in the respiratory mucosa and complications arising from myriad factors including host immune response, management, or environmental conditions (Chakraborty et al., 2014; Lacasta et al., 2008). The role of multiple pathogens forms the concept of polymicrobial diseases in man and livestock. These refer to infections by different viruses and bacteria synergism, fungi and parasites, and opportunistic infections secondary to immunosuppression (Hodgins et al., 2002). These multiple infections

may manifest as severe lesions with poor prognosis. In small ruminants, peste des petits ruminants virus (PPRV) induced pneumonia was complicated by *M. haemolytica* (Ackermann and Brogden, 2000; Emikpe and Akpavie, 2012; Gonzalez and Maheswaran, 1993; Shoo, 1989). However, the role of other bacterial complications of respiratory viral infections other than PPRV has not been well established especially in our environment.

PPRV and *M. haemolytica* co-infection may have enhanced the virulence of *M. haemolytica* in the dwarf goat (Emikpe and Ak-

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pavie, 2011). More so, the association of PPRV with other respiratory viruses and bacteria has received little attention, hence this study investigates the association of multiple viral and bacterial agents in caprine lungs from our environment. Understanding of the multiple infections will improve our knowledge on the need for multivalent vaccines for adequate control of caprine pneumonia.

Materials and Methods

Ethical approval

The study was approved by University of Ibadan Animal Care Use and Research Ethics Committee with accession number (UI-ACUREC/17/0060). The guidelines for the care and use of animals in research were strictly followed.

Study population and lung samples

This study was conducted for 72 weeks between March 2014 and August 2015, allowing for seasonal variation (wet and dry). The goats were sourced from various regions in Nigeria; 150 indigenous goats were randomly selected from a total of 700 comprising West African Dwarf, Red Sokoto and Sahelian breeds (Jarikre et al 2016).

The sex of the goats were determined physically and the age by the dentition as described by Lasisi et al. (2002). The general body conditions were evaluated and scored systemically as described by Battaglia (2001) on a scale of 1-5 (amount of fat and muscle at key anatomical points); 1=very thin (poor), 2=thin (fair), 3=normal (good), 4=fat (obese) and 5=very obese. However, all goats were scored as good, fair, or poor. All goats were screened for pulmonary lesions grossly and histologically following standard procedures as described by Jarikre et al. (2016).

Pathology and immunohistochemistry

Grossly the lungs were examined for changes in consistency, texture, color, and severity as described by Lopez (2012). Sections from all the lung lobes (apical, middle and caudal) were taken from both lungs into sample bottles with adequate volume of fixative for histological processing. The stepwise protocol for histological processing of the lung tissues was done using the automatic tissue processor with the lung tissue sectioned at a thickness of 4 μ m. Morphological diagnosis was made from each goat and the pulmonary lesions were grouped according to the morphological changes. Thin sections of the lungs were further cut, floated, and mounted on 3-aminopropyltriethoxysilane (APES) charged glass slides in quintuple with additional six sets as primary antibody controls.

Parainfluenza 3 (PI3) virus monoclonal antibody (Cat No: MA5-27876) and respiratory syncytial virus (RSV) polyclonal antibodies (Cat No: PA1-73019), were sourced from Thermo Scientific USA (thermofisher.com/antibodies). They were supplied in cold chain under optimal conditions.

PPRV, *M. haemolytica*, and *P. multocida* polyclonal antibodies were raised individually in rabbits using the PPRV lineage 1 (Nig/75) and formalin-killed whole bacteria injected with Freund's complete adjuvant (FCA), respectively, into New Zealand white rabbits in duplicates (n=6) following standard procedures. The specificity and sensitivity of the bacterial antibodies were duly verified in our previous studies (Jarikre et al., 2018).

The detection of antigens with specific antibodies in the paraffin-embedded tissues followed standard procedures. The slides were rehydrated and antigens were unmasked via heat-induced retrieval. Primary antibody concentration for each of the monoclonal antibodies was set at 4 μ g/ml while 1:100 dilutions were set for the polyclonal antibodies. The avidin-biotin-peroxidase kit (LOT: 2775482, IHC Select Detection System, HRP/DAB, Merck, Germany) was used for the staining following the instructions in the manual. The morphologically established normal goat lungs were used as negative controls, while negative sera were used as controls on the pneumonic lung tissues.

Photomicrographs of the tissues (images) were taken using a computer enabled digital AmScope camera (MU 900) and ToupView 3.2 software connected to the Olympus CX21 Microscope (Olympus Co., Tokyo, Japan).

Statistical analysis

The number and percentages of the caprine lung samples positive and negative for the three different viral antigens and the two bacterial antigens and cases of multiple infections were determined.

Results

Animals and clinical findings

In all (150), 22 goats were one-year old, 59 were two-year old, 57 were three-year old, and 12 were four-year old. Fifty-seven were from dry season (October to March) and 93 from wet season (April to August). Nine of the goats were female (7%) and 141 were males (93%). The breeds were as Red Sokoto breed (n=81), West African Dwarf breed (n=47), and Sahelian (n=22).

Clinically, the signs observed included dullness, mucopurulent oculo-nasal discharges, dyspnea, and tachypnea. Thirty-five (23%) of the goats were in good body condition, seventy-four (49%) apparently fair body condition, and forty-one (28%) in poor body condition.

Lesions and immunohistochemistry

The consolidation pattern of the lungs included cranio-ventral, lobar, and diffuse. Of the 150 goats, 61 had broncho-interstitial pneumonia (Figure 1a), 25 had interstitial pneumonia (Figure 1b), 42 had bronchopneumonia (Figure 1c), 12 had bronchiolitis, and 10 were normal caprine lung samples.

Immunohistochemically, 140 (93%) of the caprine lung tissues were positive for the antigens of the different

pathogens and 10 (7%) were negative. Viral (PPR, PI3 & RSV) antigens were detected in 113 (81%) caprine lung tissues and bacterial (*M. haemolytica* & *P. multocida*) an-

tigens were in 120 (86%) of the lungs. Thirty-seven (25%) of the caprine lung tissues were negative for the viral antigens and 30 (20%) were negative for the bacterial anti-

gens (Table 1). More than one pathogen were detected in 108/140 pneumonic goats. There were viral-bacterial antigens in 106/108 of the pneumonic goats. PPRV and *M. haemolytica* co-infection had the highest incidence (20/106); others included PI3V-*P. multocida* (18/106), PPRV-*P. multocida* (12/106), PI3V-*M. haemolytica* (10/106) and RSV-*M. haemolytica* (9/106).

PPRV was the single viral antigen (Figure 2a) in 15 (10%) animals. The antigens of other pathogens including PI3V (Figure 3a, 3b), RSV (Figure 4a, 4b), and *M. haemolytica* (Figure 5a, 5b) were demonstrated in less than five animals except for *P. multocida* antigen (Figure 6a, 6b) which was present in 19 goat lungs (Table 1). The photomicrographs of primary and secondary antibodies, and DAB controls (Figure 7a, 7b) are demonstrated.

Table 1. Name and number of agents causing pneumonia, morphological lesions, and the lesion score in 140 cases of caprine pneumonia observed in Nigeria

Agent (n)	Morphological change (lesion score)
PI3 (4)	Bronchiolar epithelial necrosis (1)
PI3 + Mh (10)	Bronchopneumonia moderate diffuse (2)
PI3 + Pm (18)	Bronchiolar epithelial necrosis (1)
PI3 + Mh + Pm (2)	Bronchopneumonia moderate diffuse (2)
RSV + Mh (9)	Bronchopneumonia moderate diffuse (2)
RSV + Pm (3)	Bronchiolar epithelial necrosis (1)
RSV + Mh + Pm (9)	Bronchopneumonia moderate diffuse (2)
PPR (15)	Bronchointerstitial pneumonia (3)
PPR + Mh (20)	Diffuse fibrinous severe bronchopneumonia (5)
PPR + Pm (12)	Bronchointerstitial pneumonia (3)
PPR + Pm + Mh (4)	Diffuse fibrinous severe bronchopneumonia (5)
PI3 + RSV + Mh (1)	Diffuse fibrinous severe bronchopneumonia (4)
PI3 + RSV + Pm (1)	Diffuse fibrinous severe bronchopneumonia (4)
PI3 + RSV + PPR (1)	Bronchopneumonia moderate diffuse (3)
PI3 + RSV + PPR + Pm (1)	Bronchointerstitial pneumonia (4)
Mh (4)	Fibrinous bronchopneumonia
Pm (18)	Bronchopneumonia moderate diffuse (2)
Mh + Pm (4)	Diffuse pleuropneumonia (5)
Total: 140	

Lesion score:

- 1= degeneration of epithelial cells
- 2= degeneration and necrosis of epithelial cells + a few neutrophils
- 3= degeneration and necrosis of epithelial cells + giant cells + a few neutrophils
- 4= degeneration and necrosis of epithelial cells + giant cells + inflammatory cells
- 5= suppurative/purulent inflammation

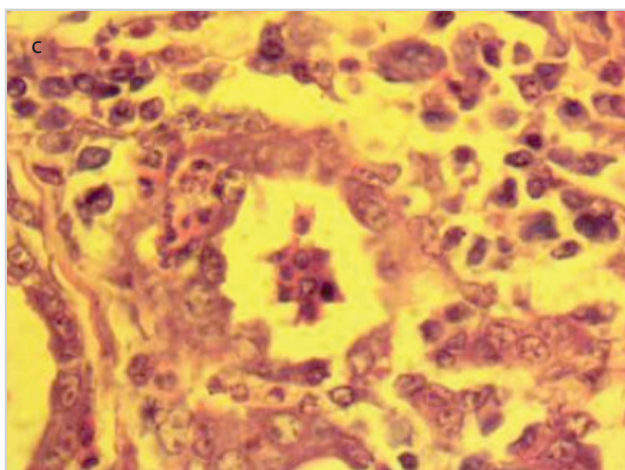
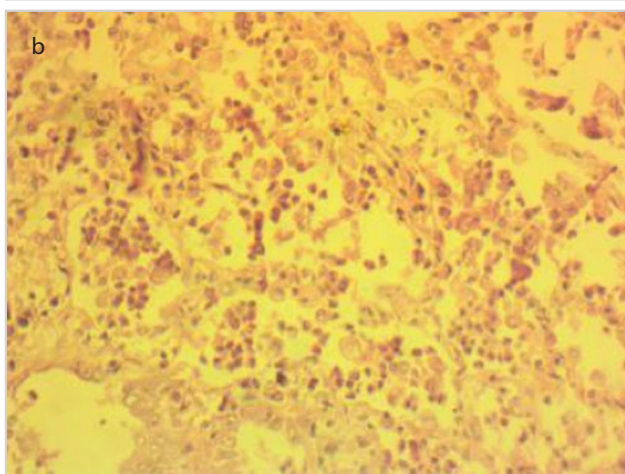
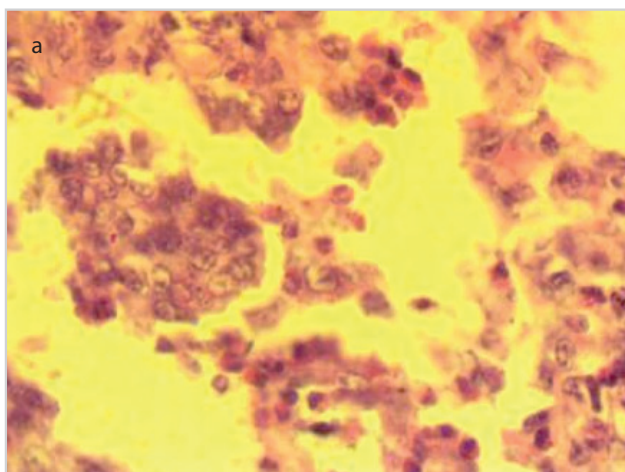


Figure 1. a-c. Photomicrographs of the lungs showing broncho-interstitial pneumonia with syncytial giant cells (a), interstitial pneumonia (b) and bronchopneumonia (c). HE x400

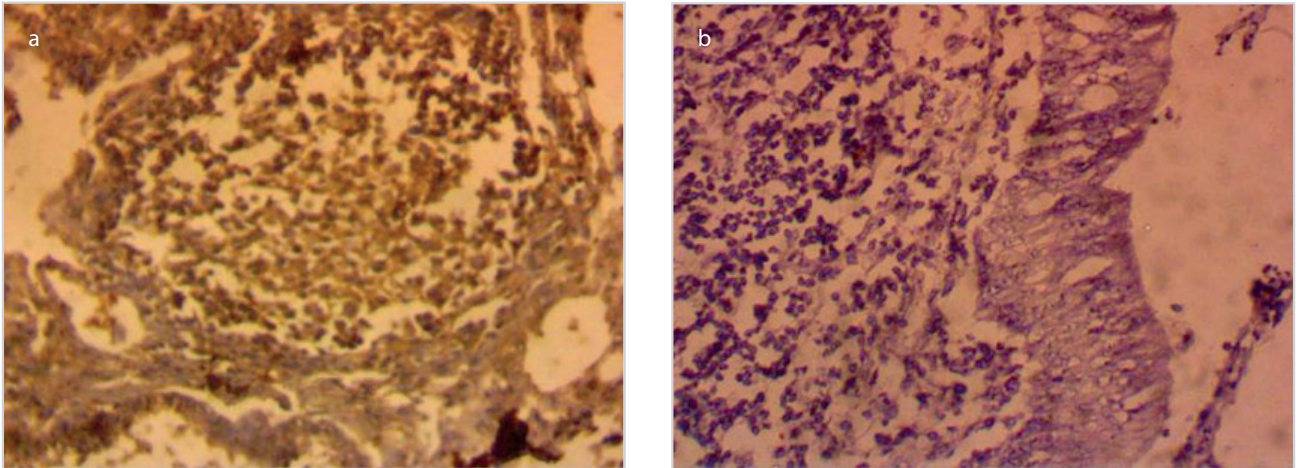


Figure 2. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to PPRV antigens (a) in bronchus-associated lymphoid tissue and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

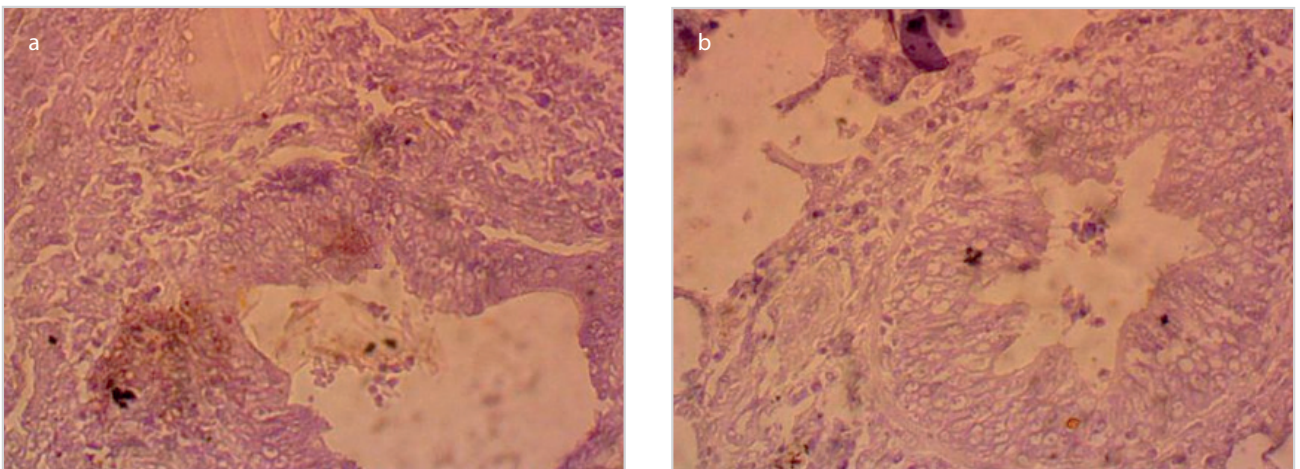


Figure 3. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to PI3V antigens (a) in hyperplastic bronchiolar epithelium and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

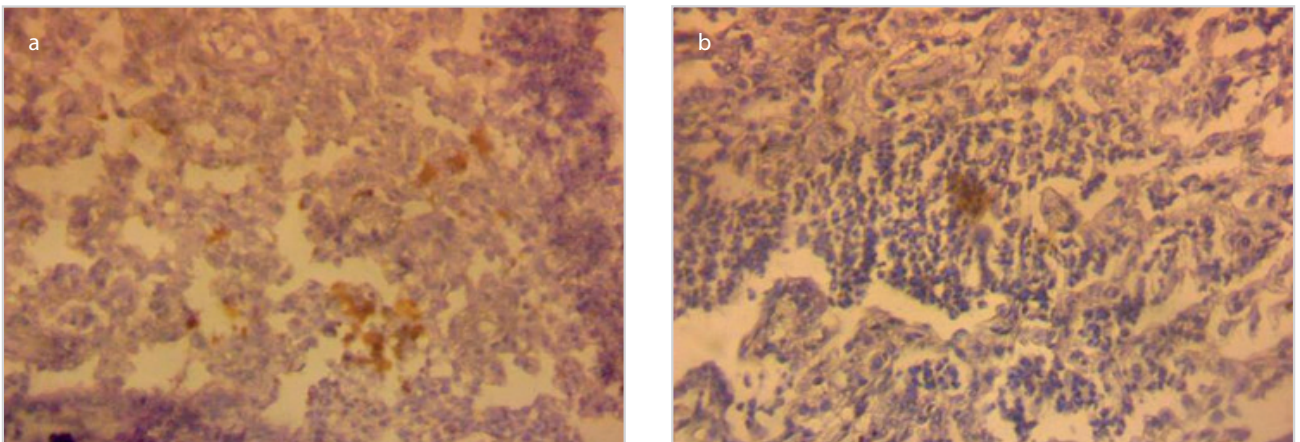


Figure 4. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to RSV antigens (a) in pneumocytes & macrophages and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

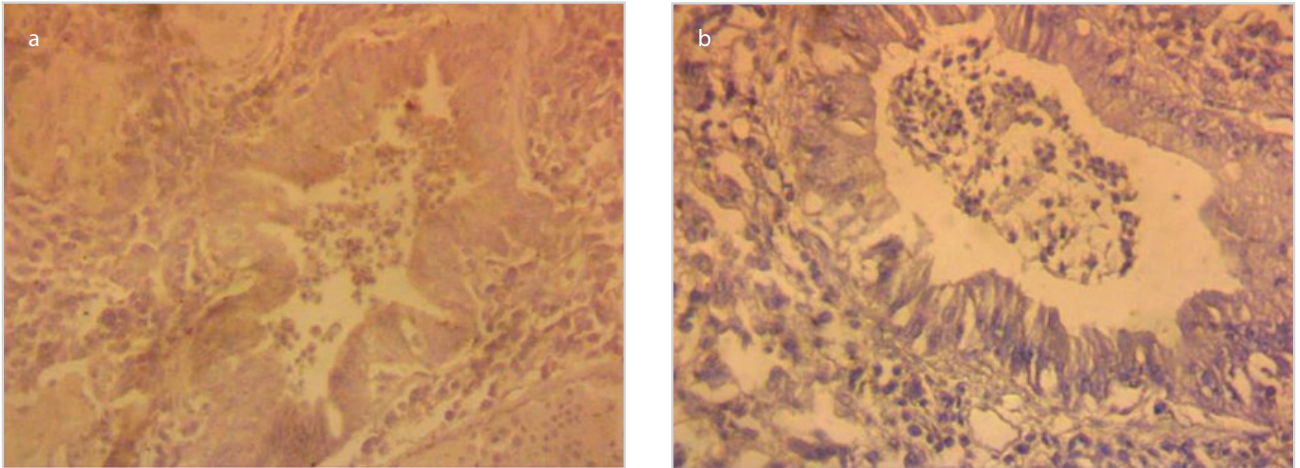


Figure 5. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to *M. haemolytica* antigens (a) in exudate and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

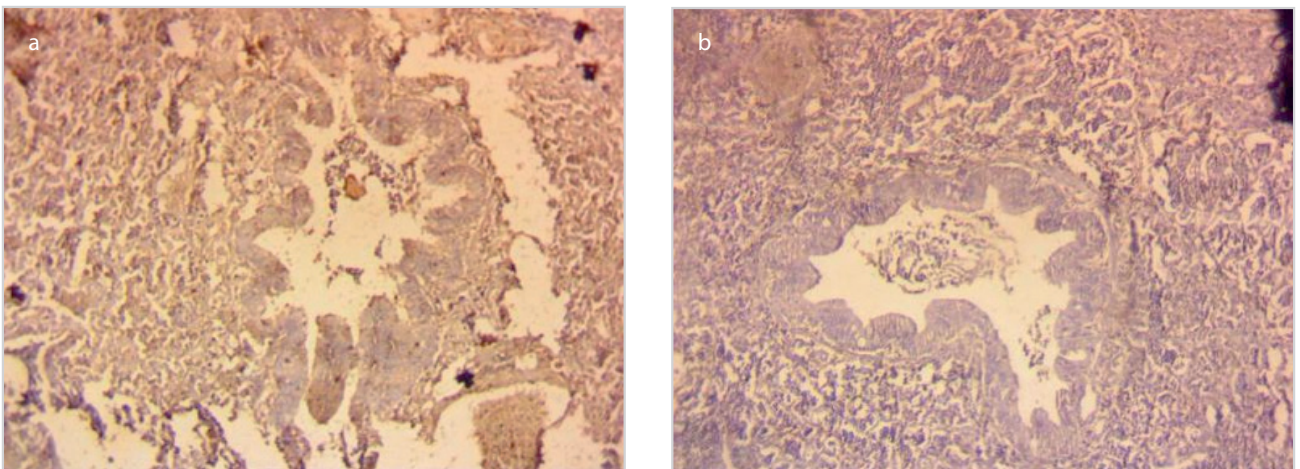


Figure 6. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to *P. multocida* antigens (a) in exudate and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

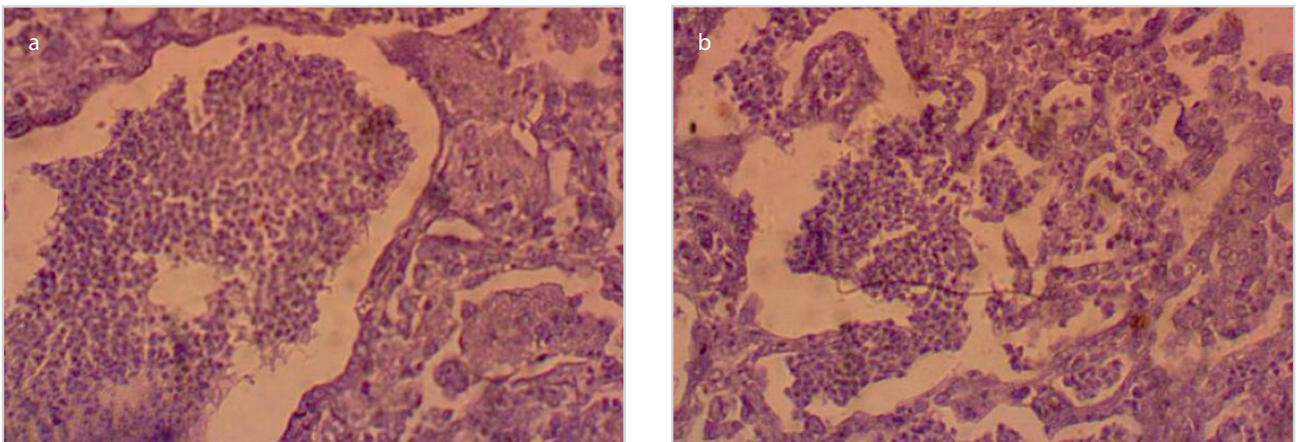


Figure 7. a, b. Photomicrograph of pneumonic goat lung used as secondary antibody (a) and DAB (b) controls. ABC HRP/ Haematoxylin counterstain x40

The specific immunostainings were characterised by the presence of light to dark brown stained antigens demonstrated in: bronchial, bronchiolar epithelial cells, macrophages, leukocytes, pneumocytes, giant cells and the desquamated bronchial and bronchiolar epithelial cells. The intensity varied with the distribution of the lesion with very slight immunostaining in the pneumocytes, interstitial macrophages, and in blood vessels; while the bronchial, bronchiolar epithelium and the luminal exudates with macrophages in the bronchial associated lymphoid tissue stained strongly. The morphological changes observed in the airways and air spaces varied from degeneration and necrosis to inflammation with presence of giant cells (Table 1).

Discussion

This study elucidates the association of respiratory viruses and bacteria in caprine pneumonia in Nigeria. It showed the prevalence of single to multiple microbial respiratory infections in goats from our environment. Single infections of PPRV, and *P. multocida* ranked highest in precipitating lesions after the dual infections of the respiratory pathogens in the pneumonic caprine lungs. This further confirms the susceptibility of goats to pneumonia as compared to sheep (Cutlip et al., 1996; Dassanayake et al., 2013; Emikpe et al., 2013), which showed that one to two respiratory pathogens are enough to produce a disease in goats. Furthermore, our findings supported the reports of Brown et al. (1991) and Saliki et al. (1994) on the role of PPRV in caprine pneumonia while also highlighting the nature or pattern of the pulmonary lesions which included broncho-interstitial respiratory viral pneumonia of goats (Kumar et al., 2004), and bronchopneumonic lesions in respiratory bacterial pneumonia in goats (Haritani et al., 1987; Yener et al., 2009).

However, it has now become evident that other pathogens other than PPRV and *M. haemolytica* are also involved in pneumonia complex of goats, which may have contributed to the endemicity of caprine pneumonia. Similar reports on PI3 virus (Fulton et al., 2000), RSV (Sharma and Woldehiwet, 1990), adenovirus (Davies et al., 1982), bovine viral diarrhoea virus (Gånheim et al., 2003), and herpes viruses (Narita et al., 2000) have indicated these agents as aetiology of pneumonia in ruminants. Recently, we also reported the detection of other respiratory viral infections other than PPR, which included PI3V and RSV in pneumonic lungs of goats in Nigeria (Jarikre and Emikpe, 2017).

It is necessary therefore to further investigate the exact interaction of these pathogens in caprine pneumonia to avoid the unnecessary abuse of antibiotics and elusiveness to control (Hodgins et al., 2002). It is worthy of note that virus-virus association, bacterium-virus association, and single infections are common in goats which further underscored the susceptible nature of goats to pneumonia.

Our findings on multiple respiratory pathogen infections in goats further agree with observations from pneumonia of alpaca neonates (Cirilo et al., 2012; Guzmán et al., 2013; Rosadio et al., 2011). The mechanisms involved in multiple pathogen infections include immunosuppression of the host due to morphological, functional or stress induced changes and colonization of pathogen to mucosa surface followed by inflammatory reactions (Hodgins et al., 2002). Respiratory viruses enable colonization of bacteria to mucosal epithelium which is followed by a severe inflammation. The influence of transport stress, helminthosis, population density, and age has been incriminated in the multiple infection phenomena (Adeyemi et al., 2017; Brogden et al., 1998; Jasni et al., 1991; Zamri-Saad et al., 1996). The high prevalence of *M. haemolytica* and *P. multocida* in the goats in this study may not be unconnected to the influence of stress and the possible reversal of virulence in the respiratory tract as highlighted in experimental pasteurellosis of goats by Zamri-Saad et al. (1989) and Zamri-Saad et al. (1991).

It is now clear that caprine pneumonia is a complex of multiple pathogens in Nigeria, a fact which may have contributed to the elusiveness of caprine pneumonia control. However, in Sub-Saharan Africa and part of Asia, the role of PPRV cannot be over emphasized (Emikpe et al., 2011; Ozkul et al., 2002).

Since vaccination is the most effective control measure; the appropriate vaccine candidate to use may be a challenge due to variation of pathogens and the different associations observed. The timing, delivery system, route and other factors are therefore also critical for optimal immune response and protection.

It is therefore important to note that different interactions occur in caprine pneumonia but the nature of these interactions need to be investigated for caprine pneumonia control. A combination of vaccines that could utilize the possible interaction mechanisms should be employed.

Ethics Committee Approval: Ethics Committee approval was received for this study from the University of Ibadan Animal Care Use and Research Ethics Committee with approval number UI-ACUREC/17/0060 on 14-07-2017.

Author Contributions: Concept – T.A.J.; Design – B.O.E.; Supervision – B.O.E.; Resources – T.A.J.; Materials – B.O.E.; Data Collection and/or Processing – T.A.J.; Analysis and/or Interpretation – T.A.J., B.O.E.; Literature Search – T.A.J.; Writing Manuscript – T.A.J.; Critical Review – B.O.E.; Other – B.O.E.

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