sciendo

DOI: 10.2478/fv-2020-0001

FOLIA VETERINARIA, 64, 1: 1—10, 2020

IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL STUDIES OF *MYCOPLASMA HYOPNEUMONIAE* STRAIN IN NATURALLY INFECTED PIGS IN NIGERIA

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ABSTRACT

Enzootic pneumonia caused by *hyopneumoniae* (MHYO) remains a serious concern to the swine industry in many countries including Nigeria. MHYO strains isolated from pigs from different countries and geographical locations are known to vary in pathogenicity. There is a paucity of information on the pathogenicity of the MHYO strain affecting pigs in Nigeria. This study investigated the pathogenicity of the MHYO strain in naturally infected pigs using immunohistochemistry and electron microscopy. Two hundred and sixty four lungs of slaughtered pigs were randomly collected from abattoirs at Abeokuta, Ibadan and Lagos, in Southwest Nigeria. A sub-sample of 104 pneumonic and 20 apparently normal lungs was selected, processed for routine histopathological examination and immunohistochemistry, while 3 lung tissues samples were selected for ultrastructural studies. The most significant microscopic changes observed were suppurative broncho-interstitial pneumonia associated with varying degrees of lymphoid hyperplasia of the bronchus-associated lymphoid tissue (BALT) and thickened alveolar septa due to cellular infiltration consisting predominantly of neutrophils and a few mononuclear cells. Immunohistochemically, MHYO antigen was detected in 86/104 (82.69 %) of MHYO-infected lung tissues and typically exhibited a granular brown reaction on the bronchial and bronchiolar epithelial lining, mononuclear cells in the BALT and luminal cellular exudates within the airways. Transmission electron microscopy revealed numerous *Mycoplasma* organisms in the lumina of the airways, in between

degenerated cilia, while a few *Mycoplasmas* were located within the alveoli. It was concluded that the MHYO strain detected in this study was pathogenic to pigs and capable of inducing pneumonia, and therefore implicated in the pathogenesis.

Key words: Immunohistochemistry; *Mycoplasma hyopneumoniae*; Nigeria; pathogenicity; pigs; pneumonia; ultrastructural changes

INTRODUCTION

Pneumonia remains one of the most challenging problems in intensive pig production systems [8, 24, 32]. *M. hyopneumoniae* is a known primary pathogen and initiator of enzootic pneumonia (EP) in pig herds [6, 22, 25, 40, 41], and contribute significantly to the development of porcine respiratory disease complex [5, 6, 22, 29, 31]. EP, a contagious, prevalent and chronic respiratory disease of pigs, constitutes a significant threat to swine health and productivity and reported to be responsible for substantial financial losses to swine producers in a number of countries [9, 20, 23, 35, 39] and has been considered as the most economically significant respiratory disease of finishing pigs [9, 39, 42]. There have been a number of publications on histopathology [15, 37] and immunohistochemical findings [37, 38] of infection with *M. hyopneumoniae* in pigs. The ultrastructural changes and the pathogenesis have also been well documented [10, 11, 17]. The appearance of lesions in different areas of the lung tissue sections has been associated with the presence of MHYO, as detected by immunohistochemistry [12, 37, 38], in-situ hybridization [18, 19], immunofluorescence [2] and transmission electron microscopy [4, 10, 36, 38]. In addition, a number of studies have demonstrated that MHYO colonizes the respiratory tract at the level of ciliated epithelial cells by attaching specifically to respiratory epithelium of the pig [10, 36, 47, 48]. The colonization of respiratory cilia by MHYO has been reported to result in ciliostasis, clumping and loss of cilia over the affected epithelium [11, 45], and colonization resulted in a significant reduction in the efficiency of the clearance of debris and the invading pathogens by the mucociliary apparatus [12]. Previous studies reported that adherence of the organism to the respiratory epithelium is a prerequisite step for colonization, pathogenicity and development of pneumonia [12, 17, 45]. This action had been shown to require a cilia binding epitome of the MHYO of which P97 adhesin (a membrane protein) has been identified and characterized [1, 15, 16, 27, 44, 48]. P97 protein has been reported to be expressed during infection with MHYO [1] and has been classified as one of the immunogens responsible for the immune response in the respiratory tract of swine [1, 16, 26, 27]. Furthermore, several studies have shown that different strains of MHYO are antigenically and genetically diverse [26] and vary in pathogenicity for pigs in different countries and geographical locations [27, 48]. Moreover, the course of EP depends on the virulence of the *M. hyopneumoniae* strain affecting the pigs [44]. This may be due to the existence of differences in virulence of the MHYO strains infecting pigs [28, 44]. Higher pathogenicity in high-virulence strain has been attributed to a high capacity to multiply in the lung tissues [36] and the induction of a more severe inflammatory process [25]. There has not been a study on the pathogenesis of EP and pathogenicity of MHYO strain affecting pigs in Nigeria. This study, therefore, investigated the pathological and ultrastructural changes in pneumonic lungs collected from slaughter-age finishing pigs with an intent to determining the pathogenicity of the MHYO strain infecting pigs in Nigeria as well as to further extend the knowledge of the pathogenesis of EP.

MATERIALS AND METHODS

Study location and sample collection

A total of 264 lung samples consisting of 60 apparently normal lungs (control group) and 204 grossly pneumonic lungs (case group) were randomly collected irrespective of the age, sex and breed from abattoirs located at Abeokuta, Ibadan and Lagos in southwest Nigeria, as well as carcasses submitted for postmortem examination at Department of Veterinary Pathology, Federal University of Agriculture, and Abeokuta. Samples were collected for a period of two years (2015—2017) on 12 occasions, 8 weeks apart. Grossly, pneumonic lungs had lesions characterized by cranioventral pulmonary consolidation (CVPC). Cases of CVPC were defined as those with pneumonic lesions affecting a minimum of three cranioventral lung lobes (i. e. the apical, cardiac and intermediate lobe). All the lung samples collected were immediately fixed in 10 % neutral

buffered formalin and left to fix for 48—72 hours. A subsample of 104 pneumonic and 20 apparently normal lungs of the formalin-fixed samples was processed for histopathological examination, while 104 samples were selected and used for immunohistochemistry.

Histopathological technique

One hundred and twenty four formalin-fixed lung tissues (104 pneumonic and 20 apparently normal lungs) were embedded in paraffin, sectioned at 3—5 μm, and stained with haematoxylin and eosin stain (H&E) for light microscopic examination to evaluate the following structures in the section: bronchi, bronchioles, bronchus- associated lymphoid tissue (BALT) and alveolar septa. The classification of histological lesions followed the semi-quantitative criteria according to Hansen et al. [15]. BALT hyperplasia was graded as mild (+) moderate (++), marked (+++) and extensive (++++).

Immunohistochemistry protocol

An immunohistochemical (IHC) test was used to detect MHYO-specific antigens on selected 104 formalin-fixed, paraffin-embedded lung tissues. Tissues were sectioned at 3—5 μm and processed for immunohistochemical staining. The IHC test was performed by the use of the heat-induced epitope retrieval technique using the citrate base antigen retrieval unmasking solution (Vector Lab., USA). Paraffin-embedded tissue sections were dewaxed by heating the unstained slides at 65 °C for 20 minutes using a hybridization incubator (Robbins Scientific® model 1000, Robbins Scientific Inc. USA). Paraffin wax was removed by washing the tissues in Hemo-De 3 times for 10 minutes each. Slides were air dried for about 20 minutes until the tissues became white. Deparaffinized tissue sections were pen-circled using PAP marker (Vector Lab., USA) and placed in antigen retrieval solution (Citra, BioGenex, CA, USA) in a plastic stander and were kept in a microwave for 20 minutes.

After cooling for about 20 minutes, slides were laid on a humid chamber, flooded with 3 % H_2O_2 (Fisher scientific®, UK) and incubated at room temperature for 15 minutes (2 times) to quench endogenous peroxidase activity. After washing 3 times (5 minutes each) in phosphate-buffer saline (PBS, pH 7.4; 0.01 M) containing 0.1 % Tween 20, sections were treated with power block, 1X blocking antibody (Universal Blocking Reagent, BioGenex, CA, USA) for 20 minutes to saturate nonspecific protein-binding sites.

After draining the excess blocking serum, sections were incubated with MHYO monoclonal antibody of 100 % specificity (Identification number D79DI-7, Dr Chris F. Minion, Iowa State University, Ames, USA) diluted to 1:500 in PBS (pH 7.4; 0.01 M) and kept in a humidified chamber at 4 °C overnight. After washing with PBS 3 times, sections were treated with biotinylated anti-mouse IgG made in goat secondary antibody (Vector Lab. Inc., CA, USA), applied at 1:250 dilution for one hour at room temperature in a humidified chamber.

The sections were washed 3 times and further treated with a labeled peroxidase-conjugated streptavidin-biotin complex (Vectastain®, Elite ABC, Vector Lab. Inc., CA, USA) for one hour.

After another PBS bath (3 times), the sections were incubated with 3,3 diaminobenzidine tetrahydrochloride (DAB) (Vector Lab. Inc., CA, USA). Sections were finally washed in running tap water, counterstained with Gill haematoxylin (Vector Lab. Inc., CA, USA), dried and covered with VWR micro cover glass (VWR®, USA). Lung tissues from known *Mycoplasma hyopneumoniae*-infected pig (known MHYO-positive) and *Mycoplasma hyopneumoniae*-free pig (known MHYO-negative) (Courtesy, Dr Uriel Blas-Machado, Athens Veterinary Diagnostic Laboratory, University of Georgia, USA) were used as positive and negative controls respectively.

Transmission electron microscopy (TEM)

TEM sample preparation was carried out at the Georgia Electron Microscopy Laboratory, University of Georgia, Athens, USA. Three paraffin-embedded lung tissue blocks (IB5, 020 and AB2) were selected for electron microscopy. From each block, tissue pieces were cut out of the paraffin-embedded blocks and excess paraffin wax trimmed off. Cut tissues were placed in glass petri dish on a hot plate to melt off excess paraffin after which they were placed in 100 % xylene overnight. Tissues samples were placed in several fresh xylene every 30 minutes for a total of 5 hours, followed by a gradual rehydration from 100 % ethanol to water. Tissues samples were processed using standard transmission electron microscopy protocol as described by Cheville and Stasko [7]. Tissues were post fixed in 1 % OsO_4 (aqueous) for 1½ hours followed by 4 rinses in deionized water; 15 minutes for each step. The tissues were dehydrated in a graded alcohol series, and then cleared in

propylene oxide before infiltration and embedment in epoxy resin (Embed 812, Electron Microscopy Sciences Hatfield, PA, USA). The embedded tissues were polymerized at 60 °C overnight. Approximately an 0.5 µm thick section was cut from each block with a glass knife, stained with Toluidine blue, and examined under light microscope to ensure that the bronchial or bronchiolar epithelium, and BALT or parenchymal tissue were present (Fig. 1). Several ultrathin sections of 50—70 nm thickness were cut on ultracut sectioning machine (Reichert Ultramicrotone, Leica Microsystems, Wetzlar, Germany) using a diamond knife (Diatome LTD, US), placed on 200-mesh CU Hex grid (Electron Microscopy Sciences Inc., Hatfield, PA, US), post-stained with ethanolic uranyl acetate and lead citrate, and examined with a JEOL JEM 1011 (JEOL Inc. Peabody, MA, US) electron microscope.

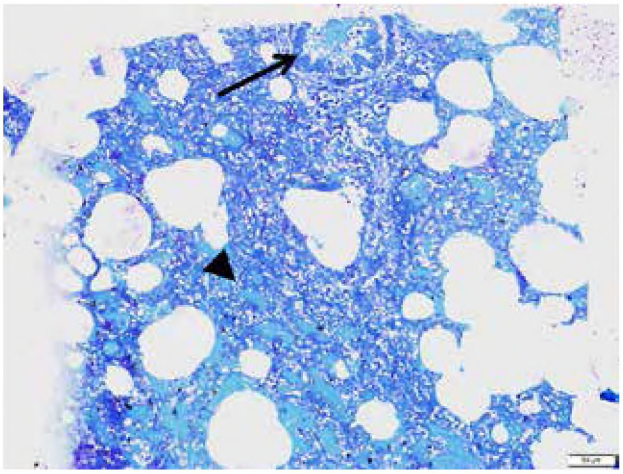

Fig. 1. Toluidine blue stain photomicrograph of 0.5 µm thick section of pig lung showing a bronchiole (arrow) and thickened alveolar septa (arrow head) Bar = 50 µm

RESULTS

Histopathological changes

Histologically, the most conspicuous lesions were massive peribronchiolar, and perivascular mononuclear cells infiltration (Fig. 2), varying degrees of lymphoid hyperplasia of BALT (mild to extensive) with formation of lymphoid nodules (Fig. 3), and thickening of alveolar septa due to cellular infiltration consisting predominantly of neutrophils with intraluminal cellular exudate (Fig. 4A). Lesions were divided into acute (8/124, 6.45 %), subacute (22/124, 17.74 %) or chronic (94/124, 75.81 %) cases of broncho-interstitial pneumonia. All acute cases were mainly suppurative broncho-interstitial pneumonia (BP) having a severe bronchitis, bronchiolitis and in some acute cases, the alveoli were collapsed and filled with inflammatory cells and oedema fluid 9 (Fig. 4B). The chronic cases were subdivided into non-suppurative (66/94, 70.21 %), and mixed (28/94, 29.79 %) broncho-interstitial pneumonia. There was moderate to severe parenchymal lymphoplasmocytic infiltration especially in the chronic stage of the infection (Fig. 5).

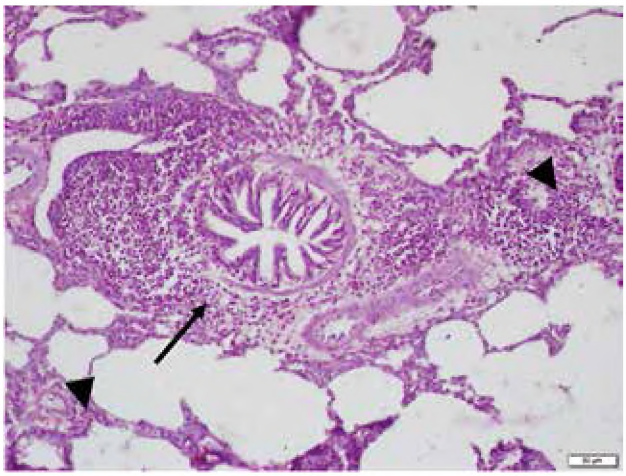

Fig. 2. Photomicrograph of pig lung section showing massive peribronchiolar (arrow), and perivascular mononuclear cells infiltration (arrow head). H&E stain; Bar = 50 µm

Immunohistochemical findings

One hundred and four lung tissue sections were selected for IHC studies. *Mycoplasma hyopneumoniae* antigens were immunolabelled as a granular brown reaction at the luminal surface of bronchial or bronchiolar epithelial cells of all positive lung tissues (Fig. 6A, 6B). There was intense immunolabelling of mononuclear cells in the BALT (Fig. 7C), and of cellular exudates within the airways (Fig. 6B).

Sections were scored ranging from 0—3 based on: no signal detectable (0), weak labelling of the ciliated epithelium lining of a least one airway (1), weak to moderate labelling on the surface of a low number of airways (2), and intense labelling on the surface of several airways (3). In total, 86/104 (82.69 %) of the lung tissue sections showed immunolabelling to MYHO antigen. Out of 86 positive lung tissues, 56 (65.12 %) showed strong immunolabelling, 14/86 (16.28 %) showed moderate labelling, 16/86 (18.60 %) weak labelling, while immunosignal was not detected in 18/104 (17.31 %) lung sections.

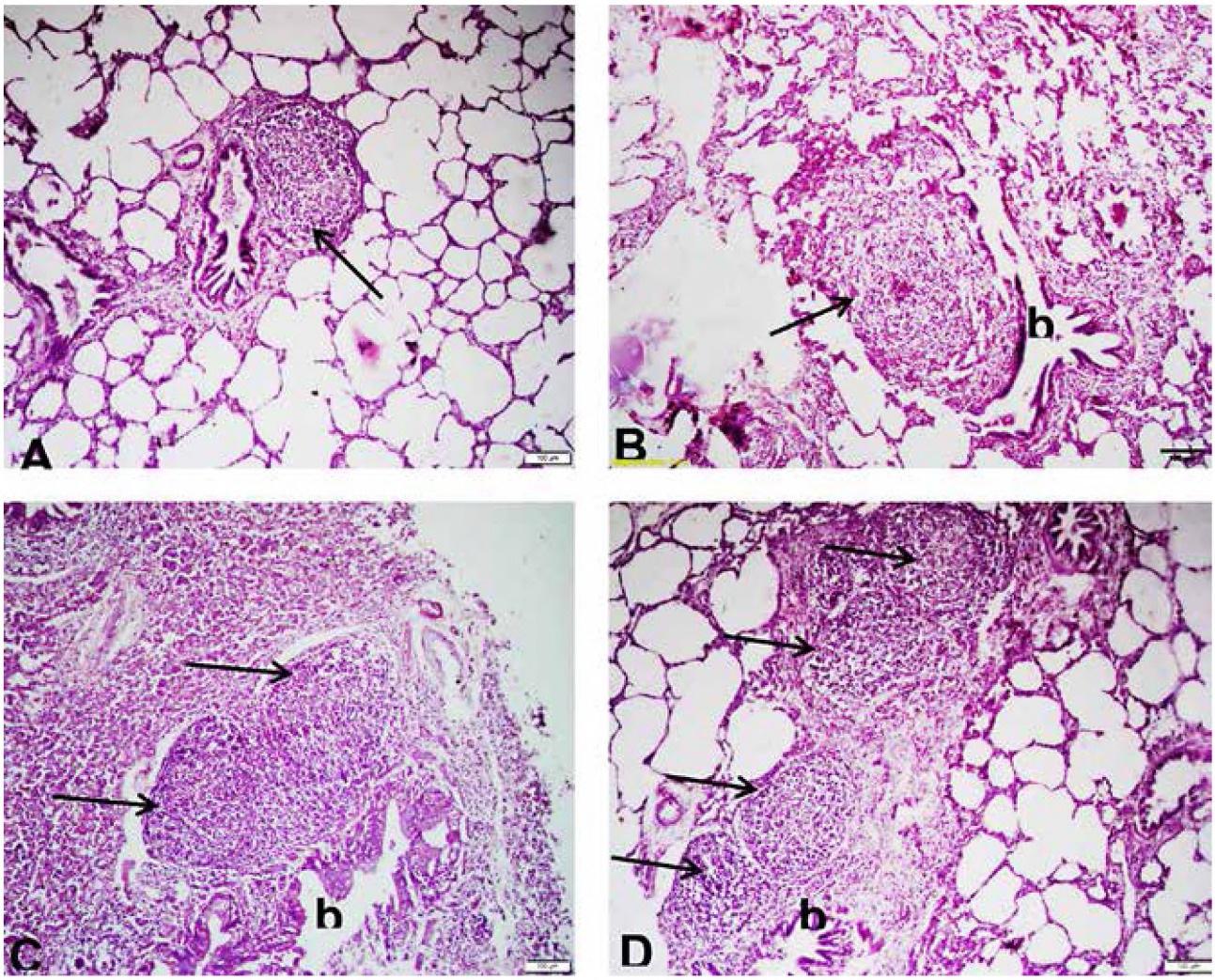

Fig. 3. Photomicrograph of pig lung sections showing varying degrees of BALT hyperplasia (A): An example of BALT hyperplasia graded as +, with lymphocytes infiltrating the muscularis mucosa of a bronchiole and formation of a lymphoid node (arrow). (B): An example of BALT hyperplasia graded as ++, with a greater diffuse infiltration of lymphocytes with formation of a bigger lymphoid node (arrow) and a markedly compressed bronchiolar lumen (b). (C): An example of BALT hyperplasia graded as +++, with the presence of a few lymphoid nodules (arrows), a compressed bronchiole (b) can be seen. (D): An example of BALT hyperplasia graded as ++++, with the presence of numerous lymphoid nodules (arrows), a large portion of the lung parenchyma is affected while a marked compressed bronchiole (b) can be seen, H&E stain, Bar = 100 µm

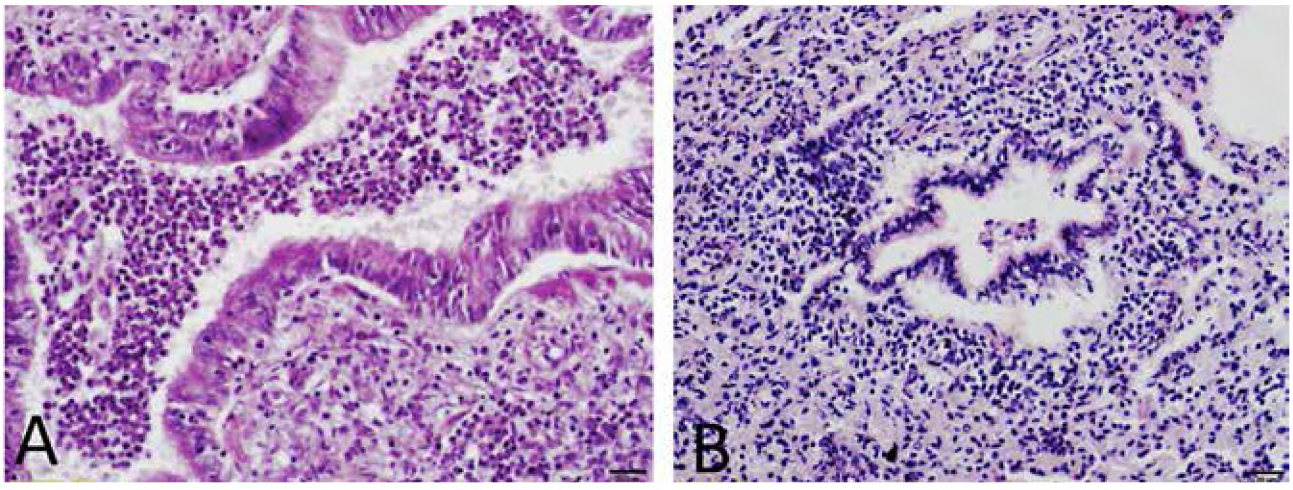

Fig. 4. Photomicrograph of a pig lung section showing suppurative bronchiolitis with concurrent epithelial hyperplasia and intra-luminal cellular exudate consisting predominantly of neutrophils and cellular debris (A). Alveoli filled with inflammatory cells and oedema fluid in the acute phase of the infection (B). H&E stain, Bar = 20 µm

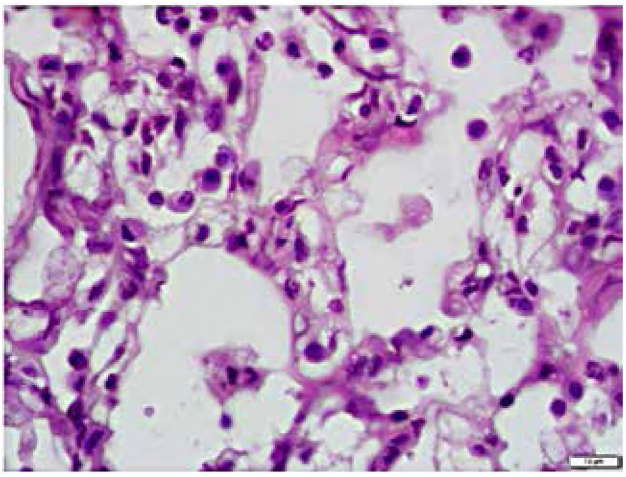

Fig. 5. Photomicrograph of a pig lung section showing interstitial pneumonia as a result of lymphoplasmocytic infiltration in the chronic stage of the infection. H&E stain, Bar = 10 µm

Ultrastructure of *Mycoplasma hyopneumoniae* and its interaction with the respiratory epithelium

In thin sections prepared from three formalin-fixed, paraffin-embedded tissue blocks, there was interaction of *Mycoplasma hyopneumoniae* with the bronchiolar and bronchial epithelial lining as observed by the electron microscopy. Numerous *Mycoplasmas* were present in the lumina of the bronchioles and bronchi. They were found mainly between degenerated cilia, but many were lying freely in the lumen and were not found in contact with the cell surface (Fig. 7). Loss of cilia over the affected epithelial cells was commonly observed. Most of the *Mycoplasmas* were oval or round in shape and varied in sizes and one was seen to be undergoing the process of binary fission (Fig. 7B, inset). A few numbers of *Mycoplasmas* were present in the alveoli (Fig. 8).

DISCUSSION

To the best of our knowledge, this is the first investigative study on the pathogenicity of MYHO strain in growing-finishing pigs in Nigeria. This study demonstrated that the histopathology of EP is complex, as nearly all pulmonary reaction patterns were observed. However, the most consistent microscopic lesion was suppurative bronchointerstitial pneumonia. The MYHO lesions were markedly influenced by secondary bacterial infections, stress, poor air quality, and also bad management [5, 13, 41]. This was characterized by peribronchial, peribronchiolar and perivascular cells infiltration consisting predominantly of

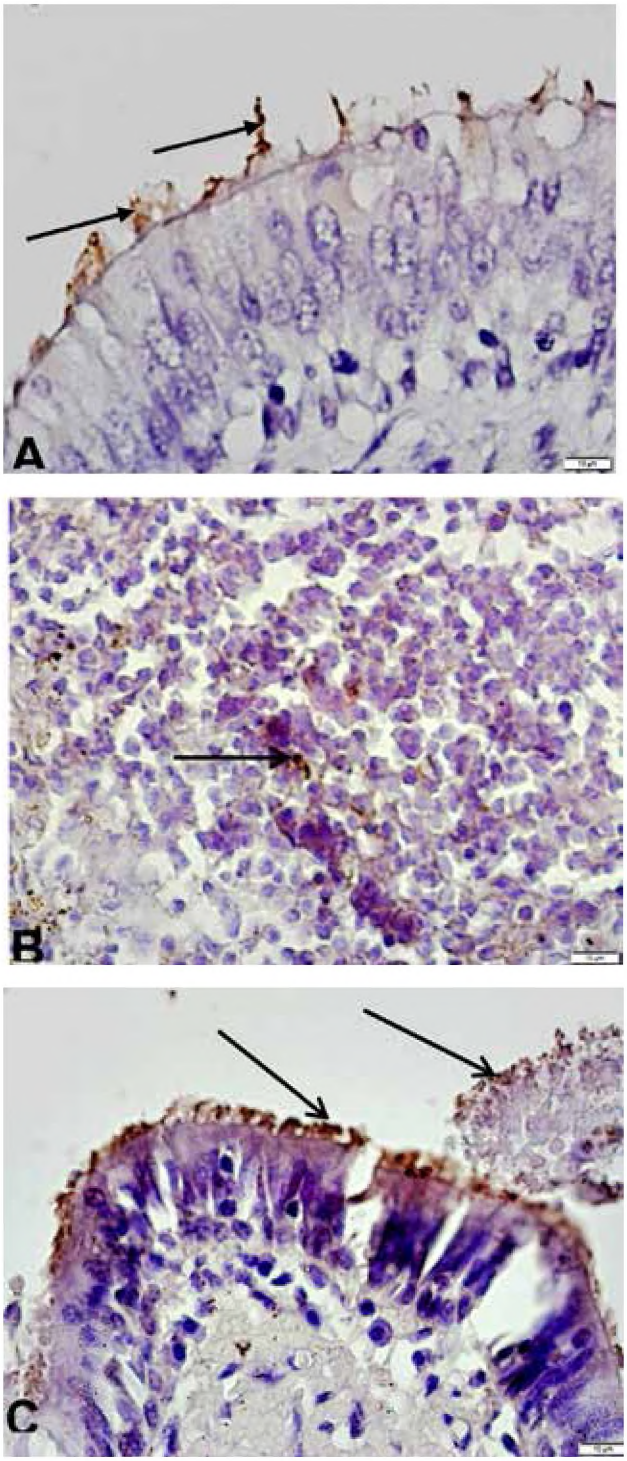

Fig. 6. Photomicrograph of pig lung sections showing immunolabelled MYHO antigen (arrows) on the apical surface of bronchiolar epithelial cells (A). IHC, Gill haematoxylin counterstain, Bar = 20 µm. Intense immunolabelling of alveolar macrophage and mononuclear cells in the BALT (arrowed) and (C) cellular exudates within airways (arrowed) (B). IHC, Gill haematoxylin counterstain. Bar = 10 µm

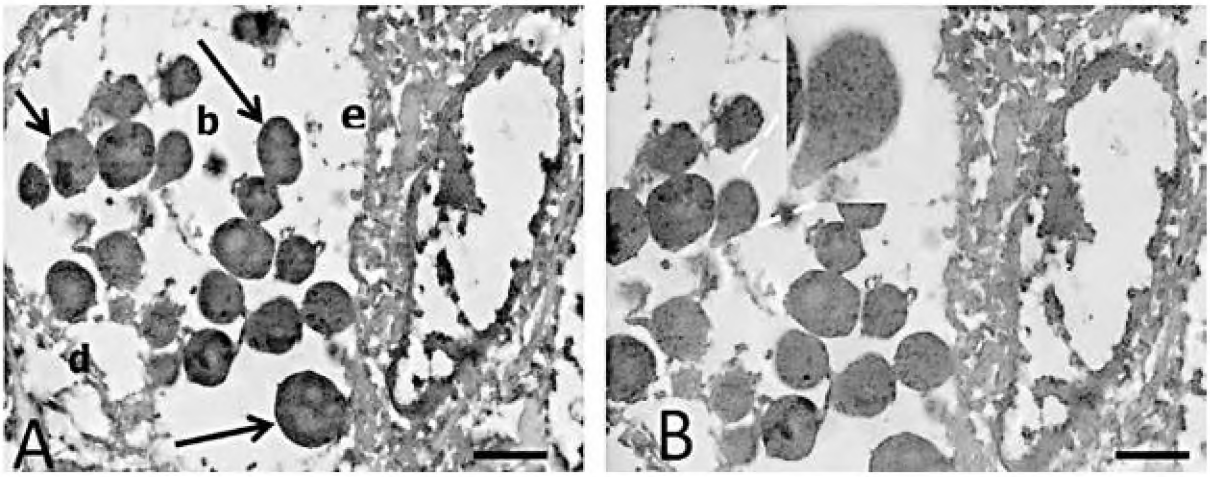

Fig. 7. Transmission Electron Micrograph (TEM) of a thin lung section of a pig showing the loss of cilia over the epithelial surface (e), degenerated cilia (d) and Mycoplasma organisms (arrowed) in the lumen of a bronchiole (b) (A). Ethanolic uranyl acetate and lead citrate stain. Bar = 500 nm. Mycoplasma undergoing the process of binary fission in the bronchiolar lumen (Fig. 7B, inset). Ethanolic uranyl acetate and lead citrate stain; Bar = 600 nm

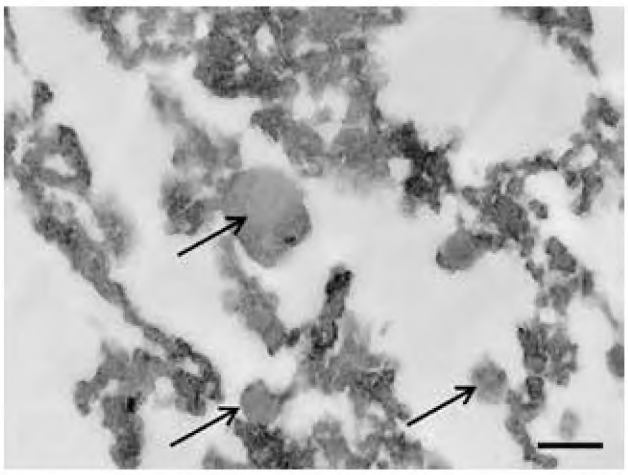

Fig. 8. TEM of the lung section showing a few Mycoplasma hyopneumonae (arrowed) within the alveoli of the infected pig. Bar = 200 nm

lymphocytes, varying degrees of lymphoid hyperplasia of the BALT with the formation of lymphoid nodules which resulted in the obliteration of the lumina of many bronchioles. There was also enlargement of alveolar septa in many cases due to the infiltration of plasma cells, lymphocytes and macrophages. These lesions have been reported by previous workers in both naturally occurring MHYO-infected pigs [18, 38] and in experimentally induced infections in pigs [8, 14, 21, 37]. The partial or complete obliteration of bronchiolar lumina associated with these lesions resulting in the collapse of surrounding alveoli, observed in this present study has been previously described [14, 23, 37]. This has been attributed to the presence of hyperplastic lymphoid aggregates [23, 37, 38], accumulation of mucus and inflammatory exudate in the bronchial and bronchiolar lumina due to decreased ciliary activities [14], increased activities of mucus secreting cells and altered glycoprotein [33] and the release of pro-inflammatory chemical mediators such as TNF-α, IL-1, IL-6 and INF-γ by alveolar macrophages [43]. The present study revealed a granular brown immunoreaction in MHYO-infected lung tissues on the luminal surfaces of bronchial and bronchiolar epithelial cells. The localization and distribution of antigens recorded in this study agrees with earlier findings obtained with an indirect immunoperoxidase method [21, 37, 38] and by in-situ hybridization [18, 19]. However, MHYO antigens were not detected in the pulmonary parenchyma in our study; this has been reported by previous workers [30]. In this study, MHYO antigens were detected in the BALT and cellular exudates in the bronchiolar lumen, which contradicts the previous report of Asai et al. [3], Kwon and Chae [18] and Kwon et al. [19]. These discrepancies may be due to infection with different strains of MHYO [18, 19]. The presence of MHYO antigens in the cellular exudates within airways recorded in this study, may suggest a local cellular immune response to MHYO infection as earlier reported [21].

A wide variety of ultrastructural changes occurring in the respiratory epithelial cells of pigs with enzootic pneumonia have been previously described [4, 10, 12]. The most significant ultrastructural change recorded in this study was a loss of cilia over the ciliated epithelial lining of the respiratory tract; *Mycoplasmas* were seen confined to the surface structure in the lumen of bronchioles and were found predominantly in the spaces between degen-

erated cilia, confirming the observation of Blanchard et al. [4]. The present ultrastructural study demonstrated and confirmed that *M. hyopneumoniae* adheres to cilia, and this adherence is associated with degenerative and/or necrotic changes and eventual loss of cilia. The exact means by which *M. hyopneumoniae* caused these cytopathic changes could not be determined by the current morphological study. However, such a change has been reported to reduce mucociliary action that would make removal and clearance of MHYO from an infected area slower and hindered thus tending to perpetuate the lesion and eventual establish the disease and its lesions [12, 17] as well as rendering the tract susceptible to secondary bacterial infection [10, 11, 17]. The study of Park et al. [33] had earlier elucidated the pathogenic mechanism of *Mycoplasma hyopneumoniae*-infected pigs and reported that the organism altered and increased fucosyl glycoconjugate in *M. hyopneumoniae*-infected pigs as a potential factor that enhanced colonization and adherence of *Pasteurella multocida* type A in bronchial and bronchiolar ciliated epithelial cells which led to enzootic pneumonia in infected pigs.

In this study, a few numbers of *M. hyopneumoniae* were present within the alveoli; this is in contrast to the previous report of Irigoyen et al. [17] who noted that *Mycoplasmas* are rarely present in the alveoli. However, this result supports a recent report of Raymond et al. [36] that showed that approximately 8% of *M. hyopneumoniae* cells reside intracellularly within the porcine epithelial cells where the organism was reported to associate with integrin β1 on the surface of epithelial cells via interactions with surface-bound fibronectin and initiates signaling events that stimulate pathogen uptake into clathrin-coated vesicles (CCVs) and caveosomes. These early events thus allow *M. hyopneumoniae* to exploit an intracellular lifestyle by commandeering the endosomal pathway [36]. Such discrepancies may be due to infection with a highly virulent and pathogenic strain of MHYO which has a high capacity to multiply in the lung tissues [34]. Differences in virulence could also be due to variation in the expression levels of virulence-associated genes like adhesins [36]. It is plausible to suggest from this observation that the alveolar lesions recorded in this study may be due to direct pathogenic action of the *M. hyopneumoniae* that resulted in severe structural and functional impairment of the respiratory tissues as a result of a severe inflammatory response.

CONCLUSIONS

In the present study, the localization and distribution of *M. hyopneumonia* antigens in the lung tissues reported in this study provided some insight into the pathogenesis of enzootic pneumonia, investigated the pathogenicity of MHYO strain affecting pigs in Nigeria and further expounded the pathogenesis of enzootic pneumonia in pigs.

Therefore, it is concluded that the MHYO strain detected in this study induced pulmonary pathology including broncho-interstitial pneumonia and loss of cilia over the ciliated epithelial cells of the airways. These have been reported to be prerequisites for the development of pneumonic lesions. Further studies are warranted particularly a molecular study of the MYHO strain detected in this study.

ACKNOWLEDGEMENTS

This study was supported by a grant received from Nigerian Tertiary Education Trust Fund (TETFund) AST&D Grant, No: FUNAAB/CENIP/AST&D/Not./01 through the Federal University of Agriculture, Abeokuta, Nigeria, awarded to Dr M. O. Olaniyi. The authors would also like to acknowledge Prof. S. O. Akpavie for excellent review of the manuscript and Mr. Apantaku James technical assistance.

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Received May 29, 2019
Accepted November 14, 2019