IJMPR 2022, 6(4), 01-07

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article

SJIF Impact Factor: 5.273

ISOLATION, MOLECULAR AND PATHOLOGICAL INVESTIGATION OF CONTAGIOUS BOVINE PLEUROPNEUMONIA IN KHARTOUM STATE, SUDAN

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Received on: 01/03/2022
Revised on: 21/03/2022
Accepted on: 11/04/2022

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ABSTRACT

This study was carried out to update the occurrence of Contagious Bovine Pleuropneumonia (CBPP) in Khartoum State using isolation, molecular techniques and DNA sequencing of the causative agent. In addition to that tissues had been subjected to histopathological examination to the target sections (lungs). *Mycoplasma mycoides subsp mycoides (Mmm)* strain was isolated and characterized by previous methods. Using bioinformatic tools the isolated (RH) strain was confirmed to be (*Mmm)* and 100% similar to certain strains in gene-bank (PG1andVmm). Typical gross lesions of CBPP observed in tissue samples including expanded interlobular septa, hepatization, and fibrinonecrotic exudations were observed. Furthermore histopathological sections showed typical lesions. The sections revealed thickening of the pleura with fibrinous tissue mixed with homogenous eaosinophilic material. The alveoli are filled with oedema, fibrin with emphysema. Some dry areas showed fibrosis which is indicative of fibrinous pneumonia. These findings updated the presence of CBPP in Khartoum state using isolation and identification of the causative agent with Histopathological picture

KEYWORDS: Contagious Bovine Pleuropneumonia, *Mycoplasma mycoides subsp mycoides*, Khartoum state.

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is caused by *Mycoplasma mycoides subs mycoides*).[1,2] It was the only bacterial disease in List "A" Disease grouping of the World Organization for Animal Health (OIE) which requiring urgent outbreak reporting.[3] The disease is an important and economically affects the cattle production throughout most of sub-Saharan Africa.[4] It has an economic importance because of its mortality and production losses as a result of high disease control cost and restriction of cattle trade.[5] The disease is transmitted by direct contact between infected and susceptible cattle.[6,7] The clinical manifestations in cattle ranged from hyperacute through acute, subacute and chronic forms. CBPP is characterized by anorexia, fever and respiratory signs, such as dyspnoea, polypnoea, cough and nasal discharges during the acute stage of the disease when the causative agent can spread rapidly; in the chronic stage there may be long-term persistence of the agent.[2] The main signs in calves associated with the disease are joint swelling lesions.[8] The clinical picture of the disease is associated with the development of thoracic lesions, especially the hepatization of the lungs and severe pleurisy particularly during the acute stage of the disease and the formation of pulmonary "sequestra" in chronic cases.[9] Macropathologically (CBPP) is characterized by fibrinous exudative pleuristy, enlarged interlobular septa and marbled appearance. The CBPP lesions are bronchiolar necrosis and oedema which progress rapidly to an exudative serofibrinous bronchiolitis with extension to the alveoli and uptake of alveolar fluid into tissue spaces. The lymph vessels are ultimately septal lymphatics.[10] The causative agent (*MmmSC*) needs selective media to grow and identified as a silky, fragile filament called (comet) in broth and classical appearance 'fried eggs' on agar media.[11] The molecular typing identifies *Mycoplasma mycoides* cluster and *M mm* at the molecular sizes of 574bp as well as 275 bp respectively.[12] In Sudan CBPP is considered the most serious disease of economic importance, which adversely affects foreign trade.[13,14] CBPP has been enzootic since the beginning of this century. It is considered one of the most serious diseases of cattle in Sudan, leading to economical losses in forms of debilitation and death of sick animals and adversely affecting cattle trade.[13,15] Khartoum State is a large market and receives animals from all over the country. Hence surveillance and data collection should be a priority to quickly detect infected and carriers' animals. The current research was directed to illustrate the actual picture of CBPP in Khartoum State by isolation and identification of the causative agent using conventional and Molecular technique and Histopathological sections were explained.

MATERIALS AND METHODS

Samples collection

Specimens were cut and placed in sterile plastic bags, labeled and transported directly at 4ºC to the Central Veterinary Research Laboratory (Sudan) ; Mycoplasma department. Samples were also taken for histopathology; small pieces (about 2~ 3 cm) representing the tissue was fixed in 10% formal saline.

Media

Gourlay's basic medium contains: Bacto tryptose (Oxoid) supplemented with yeast extract, horse serum, glucose, in addition to penicillin and thallium acetate was used in the study. The medium was used as broth or solid media.

Method of isolation

Lung tissue samples were pulverized in broth media. The suspension was then tenfold diluted up to 3 to 10 tubes; to minimize contaminating bacteria. Dilutions were placed on sold media.

Identification of the isolates

The identification was performed using cultural characteristics, Biochemical tests, Serological tests and molecular tests.[2]

Molecular characterization

DNA extraction

DNA was extracted from the mycoplasma culture using the Guanidine chloroform method as described by.[16,17]

PCR reaction using *M. mycoides* cluster primer pairs

A pair of primers was used according to[18] method. Their sequences are:

MYC1-F: 5'-TTCTAAATTAGTTACTCGTGCA-3'
MYC2-R: 5'-AATAAACTGTATTCTCTAGCCA-3'.

PCR reaction using *M. mycoides* specific primer pairs

Amplification of the MSC gene of the mycoplasma genomic was done using primers:

MSC-F: 5'ATACTTCTGTTCTAGTAATATG3'
MSC-R: 5'CTGATTATGATGACAGTGGTCA3'.

PCR amplify band size of 275 bp. PCR was also performed using the one step (single tube) in a 25µl final volume using iNtRON's Maxime PCR PreMix Kit (iNtRON i-Taq, South Korea).

Visualization of PCR products

After PCR amplification of each gene, the PCR products were separated on a 2% agarose gel (iNtRON biotechnology, South Korea).The PCR product was visualized by ultraviolet trans-illuminator (Bio.Doc-it UVP, Cambridge, UK). The molecular weight of DNA bands was estimated in relation to standard 100bp DNA ladder.

Sequencing of 16sRNA gene

DNA purification and standard sequencing was performed by Macrogen Company (Seoul, Korea).

Bioinformatics analysis

The chromatogram was viewed by Finch TV program,(http://www.geospiza.com/Products/finchtv.shtml). Then the nucleotides sequences were searched for sequences similarity using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi.).[19] Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using BioEdit software.[20]

Histopathology: The target lung tissue samples, fixed into 10% Formalin and prepared for histopathological examination according to Bancroft and Layton.[21]

RESULTS

Isolation of *Mmm* (SC)

Mycoplasma isolate was recovered from pneumonic and pleural fluid showing typical clinical signs such as; excess mucous secretion from nostrils and difficulties in breathing (Fig.1). The post mortem showed there were adhesions to the chest, fibrinous tissue on thoracic ribs and hepitization of lung with marbled appearance Figures (2 and 3). Typical gross lesions of CBPP observed in tissue samples including expanded interlobular septa, hepatization, and fibrinonecrotic exudation.The RH local strain was successfully isolated and identified as *Mycoplasma mycoides* subsp. *mycoides* depending on colony characteristics; fried-egg appearance of colony under low power lens of stereomicroscope was observed (Figure 4). In the broth cultures short and long filaments appeared using dark field microscopy. Digitonin test was positive Figure (5), there was an inhibitory zone around the digitonin saturated disc; this test confirmed that the isolate is Mycoplasma. Biochemical tests indicated that the isolate was positive for glucose fermentation test; it changed the color from red to yellow, positive for tetrazolium reduction where the color changed to brick-red and negative for arginine hydrolysis where there was no color change (Figure 6). Growth inhibition test revealed inhibitory zone without colonies encircling of the disk saturated with specific *Mmm* antisera (Figure 7).

Figure (1): Exess mucus secretion from nostrils-Acute stage of CBPP disease.

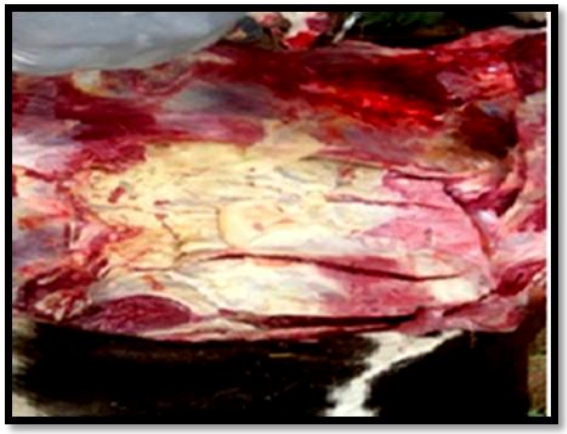

Figure (2): Chest lesions of CBPP showing fibrinous tissue on thoracic ribs of CBPP diseased animal.

Figure (3): Hepitization and marbled appearance of CBPP infected lung.

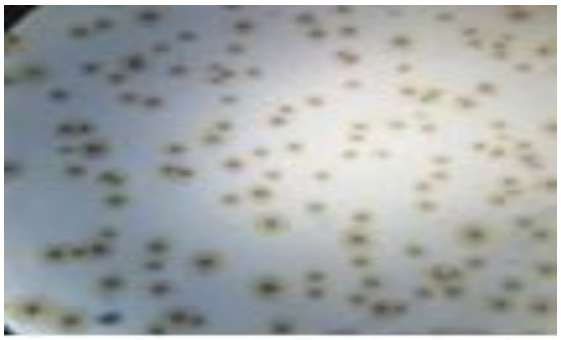

Figure (4): *Mycoplasma mycoides* sub sp. *Mycoides* colonies showing fried egg appearance.

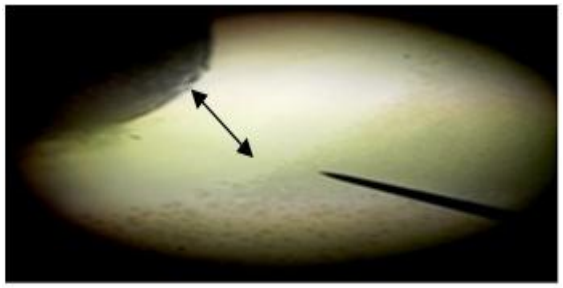

Figure (5). Digitonin test: The isolate showed inhibitory zone around the digitonin disk.

Figure (6): Biochemical tests for *Mycoplasma mycoides* sub sp *mycoides* isolate.

T: Tetrazolium reduction test
A: Araginine hydrolysis test
G: Glucose fermentation test

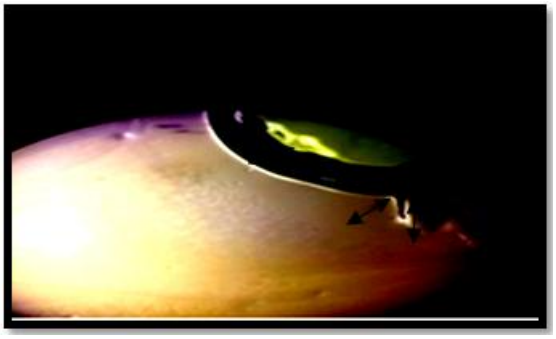

Figure (7): Growth inhibition test showing inhibitory zone around a well filled with specific antiserum against CBPP.

Molecular test using cluster primer pairs MYC1/MYC2, the RH isolate showed fragment band size on Agarose 574 b.p (Figure 8).Using specific primer pair's MSC1/MSC2, the isolate showed fragment band size on agarose gel, 275 b.p (Figure 9).

The DNA sequencing for (RH) *M. mycoides subsp. mycoides* strain showed 100% overall identity to two strains the *Mmm* PG1 and *Mmm* Vmm gene strains from gene- bank (Figure 10).

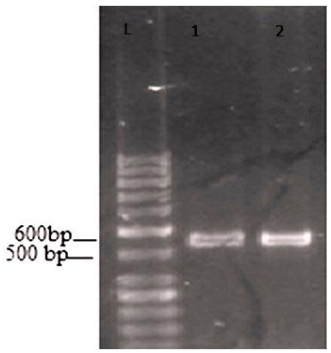

Figure (8): PCR reaction using *Mycoplasma mycoides* cluster primer.Lane: DNA ladder 100 bp.Lane1: positive control. Lane2: RH local isolate.

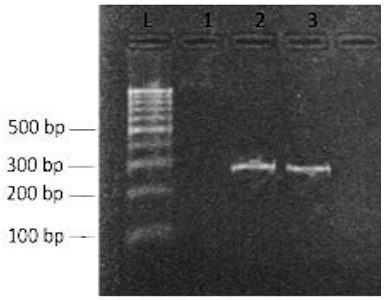

Figure (9): PCR reaction using *Mycoplasma mycoides* specific primer.L: DNA ladder 100 bp. Lane1: Negative control, Lane2: positive control, Lane3: RH local isolate.

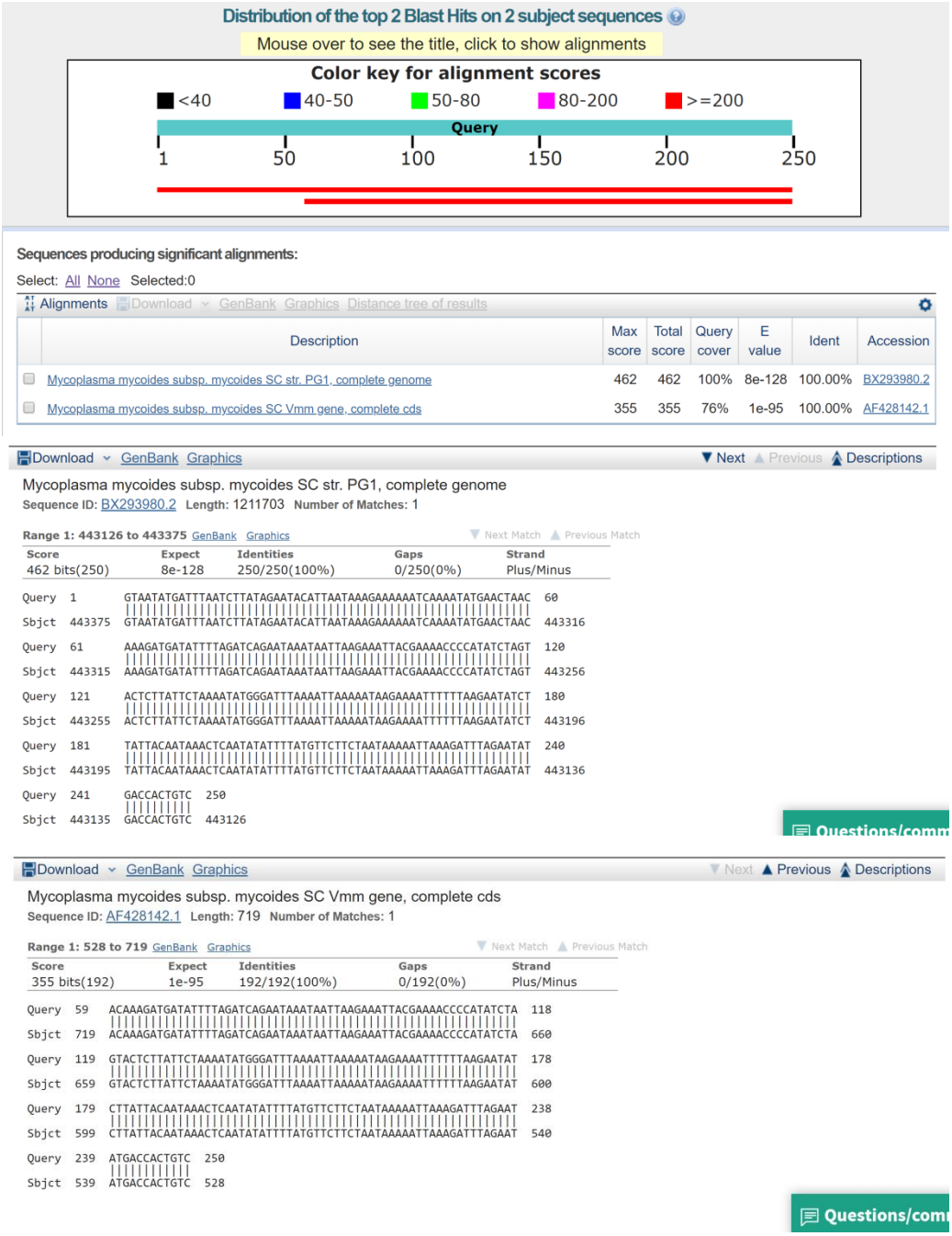

Figure (10): Bioinformatic results showing 100% overall identity to the *Mmm* PG1and *Mmm* Vmm gene strains from gene-bank.

Histo pathological sections from positive samples showed thickening of the pleura with fibrinous tissue mixed with homogenous eaosinophilic material. The alveoli are filled with oedema and fibrin with emphysema.

Moderate accumulation of mononuclear cell as well as exudates in the alveolar septae. Some dry areas showed fibrosis which is indicative of fibrinous pneumonia. Different histopathological pictures shown from *Mmm* SC-type isolate were recovered. Figures (11, 12, 13 and 14).

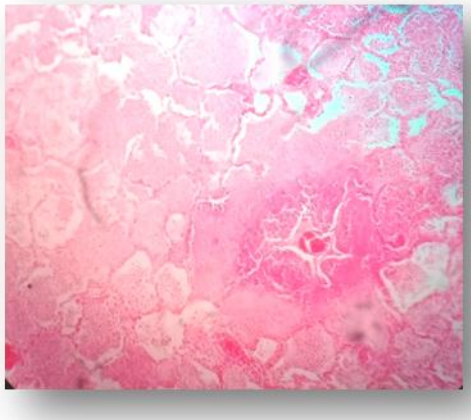

Figure (11): shows severe pleuritis diffusion with extensive fibrosis (fibrinous pneumonia) (H&E ×100).

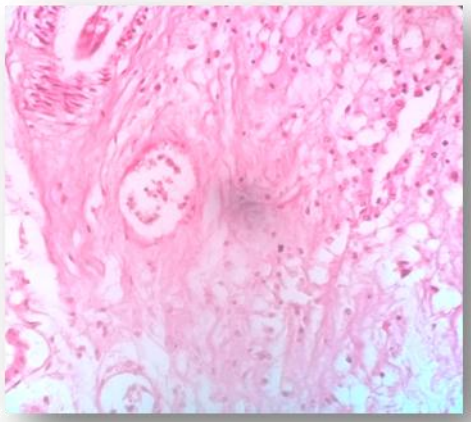

Figure (12): An interstitial inflammatory infiltrate composed of neutrophilic, eosinophilic and mononuclear cells granulocytes within the interstitial space of the lung. (H&E ×100).

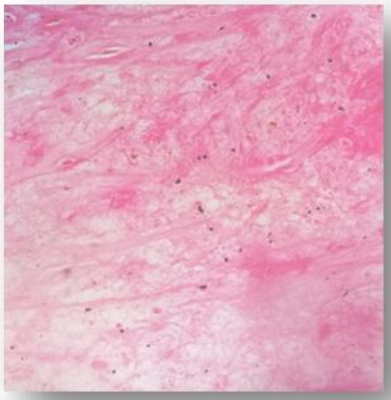

Figure (13): Fibrinous pleurisy: thickening and inflammation of the pleura (H&E ×100).

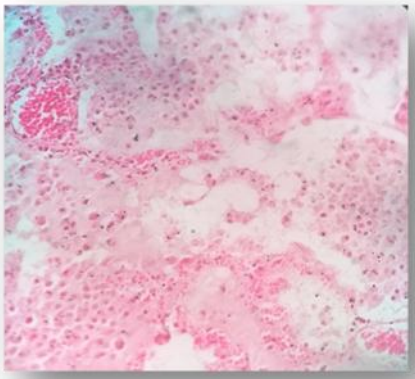

Figure (14): Heamorage and thickining of alveolar septae with fibrin and odema exduates and WBCs (H&E ×100).

DISCUSSION

CBPP is one of the major infectious diseases which affecting cattle in Africa. It was found to be an important disease in Sudan according to the last Seroprevalence in Sudan (2016) in which the disease Seroprevalence ranged between 1.7 to 26.7% in different states (Surveillance of Trade Sensitive Diseases project -STSD project sponsored by African Union).

In the present study an isolate of *Mmm* was recovered from an outbreak in dairy farm at Soba region. The animals showed typical acute clinical signs including nasal discharge, shallow and rapid respiration and cough which are typical symptoms of the disease as reported in.[2] The postmortem findings were adhesions to the chest and the lung showed red color and hepitization.

The isolates were identified as *Mmm* based on microscopic appearance. The broth culture was investigated by dark field microscopy; short and long filaments indicated the presence of Mycoplasma.[22,23,24] The colonies were small, 1 mm in diameter; with the

classical 'fried-egg' appearance.[25,2] The isolates were inhibited by digitonin which differentiate mycoplasma from acholeplasma.[23,26,27] based on their cholesterol dependency. The isolate was positive for glucose fermentation test and tetrazolium reduction and negative for arginine hydrolysis, this result is in agreement of Ameera and OIE manual.[27,2] The growth inhibition test (GIT) is recommended by the OIE manual. The present result revealed a small inhibition zone around the disk as Isam.[23] results. The small inhibitory zone can be attributed to many factors, Wallace and Clyde.[28] reported that the inhibition does not consist always of sharply defined zone surrounding antiserum disks. Strain variation and antiserum quality in addition to inoculums size and titer, affect on it; generally, any clear-cut suppression of growth can be considered as a positive result. The PCR has confirmed the previous suspicion. The PCR test approved the isolate is *Mmm* as previously described by.[2,27] manual. This finding supported the isolation results and gave additional evidence for the diagnosis of the disease. Bio informatics results showed high similarity to two strains in gene-bank which confirmed that it is *Mmm* strain.

Accurate gross lesions of lung samples were pleurisy (acute or chronic), consolidation, marbling or thickening of interlobular septa, red or grey hepatization, and fibrosis as previously described by.[9] The histopathological sections of the target tissues revealed typical histopathological picture of CBPP in acute stage. Thickening of the pleura with fibrinous tissue and alveoli are filled with oedema and fibrin with emphysema. Some dry areas showed fibrosis which is indicative of fibrinous pneumonia. Isam.[23] studied the disease in Khartoum state and found typical pictures of the CBPP which include fibrinonecrotic pneumonia within filtration of inflammatory cells and fibrin and distention of interlobular septae. Also (Jubb and Kenndes,[29] found there was presence of lymphocytes and alveolar macrophages around the lymphatic vessels. Our results are similar to findings of Li *et al.*[30] who reported the accumulation of inflammatory cells in alveoli, bronchioles, necrotic debris in bronchiolar lumen, disruption of ciliated epithelium of bronchioles and pulmonary congestion in lung samples of CBPP infected cattle. These findings supported the isolation results and gave additional evidence for the diagnosis of the disease.

CONCLUSION

This study confirmed and updated the presence of CBPP in Khartoum state by isolation and identification of the causative agent using conventional and Molecular technique and Histopathological sections.

Conflict of interest statement

The authors have declared that no competing interests exist.

ACKNOWLEDGEMENTS

This work was carried out at Department of Mycoplasma, Central Veterinary Research Laboratory, Soba (Animal Resources Research Corporation ARRC).

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