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Isolation and identification of *Mycoplasma mycoides* subsp. *mycoides* in cattle from south-east Nigeria

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Abstract

Background: *Mycoplasma mycoides* subsp. *mycoides* is the causative organism of Contagious Bovine Pleuropneumonia (CBPP). It is a trans-boundary disease and an endemic in Nigeria having caused serious financial loss for the country's economy.

Aim: This study was undertaken to isolate and confirm the presence of *M. mycoides* subsp. *mycoides* (*Mmm*) in cattle, from three selected South-Eastern states of Nigeria.

Method: A total of 90 bovine samples (25 pleural fluids and 65 lung tissues) suggestive of CBPP were collected from different abattoirs in the three selected South-eastern states of Nigeria (Anambra, Enugu, and Imo), for the isolation of *Mmm* by employing cultural method, whereas for confirmation polymerase chain reaction (PCR) approach was used. The collected samples were cultured on Pleuropneumonia like organism (PPLO) agar according to specific protocols.

Results: Twenty five of the samples (lungs and pleural fluid) were positive for *Mmm* on PPLO agar giving an isolation rate of 27.7%. Only 21 of the isolates were further confirmed using PCR. The PCR amplification of the isolates produced a product of 1.1 kbp which is specific for *Mmm*. No positive isolates were recovered from Imo state.

Conclusion: This study confirms the presence of *Mmm* as the causative organism of CBPP in Southeast Nigeria. It is recommended that active surveillance and vaccination protocol should be undertaken in the region for the control and prevention of this disease.

Keywords: Cattle; CBPP; *Mycoplasma mycoides* subsp. *mycoides*; Polymerase chain reaction.

Introduction

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the etiological agent of Contagious Bovine Pleuropneumonia (CBPP) (Thiaucourt *et al*., 2006; Campbell, 2015). It is a highly contagious and fatal disease of cattle, with serious financial implication in Nigeria (Fadiga *et al*., 2013). CBPP is a trans-boundary disease and has been classified as one of the bacterial diseases in OIE's list A diseases (OIE, 1997; Litamoi *et al*., 2004).

CBPP can manifest in hyper-acute form, where it is usually fatal and most times no clinical signs are observed (Thiaucourt *et al*., 2006). The incubatory period of this disease is relatively long with an inconsistent clinical course (Litamoi *et al*., 2004). Clinical signs of the disease include anorexia, fever, dyspnea, cough, and nasal discharges (Provost *et al*., 1987). Infections in adult cattle are most of the time restricted to the respiratory tract, while in calves, the disease usually manifests as arthritis (Litamoi *et al*., 2004; Campbell, 2015). Several molecular tools and technologies have been developed for the identification and confirmation of the causative organism (Dedieu *et al*., 1994; Lorenzon *et al*., 2002; Miles *et al*., 2006). Miles *et al.* (2006) developed a polymerase chain reaction (PCR) technique that could differentiate *Mycoplasma* species based on their origin. The PCR method is quite efficient and specific. The United States of America and Australia have eradicated CBPP through strict control of cattle movement, prohibiting large scale slaughter, and financial compensation to owners. This has, however, become difficult in Nigeria due to several factors, such as poor implementation of the test and slaughter policy, unrestricted movement of nomads across state boundaries which has made accurate monitoring of CBPP difficult (Egwu *et al*., 1996; Chima *et al*., 2001). Several authors have documented the outbreaks, prevalence, and economic importance of the disease in Nigeria (Fadiga *et al*., 2013; Ankeli *et al*., 2017; Jasini *et al*., 2016).

In Africa, the application of the stamping out policy for the eradication of CBPP has faced so many challenges.

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Consequently, the control of the disease has depended heavily on the use of the vaccine (T1/44 or T1-SR) (Thiaucourt *et al*., 2000; Litamoi *et al*., 2004). National Veterinary Research Institute (NVRI) Vom, produces CBPP vaccine (T1/44) in Nigeria (NVRI, 2007). However, this vaccine has some drawbacks (Egwu *et al*., 1996; Thiaucourt *et al*., 2006).

Presently, in Nigeria, there is little or no information on the status of this disease in South-East Nigeria as many studies have been centered within Northern Nigeria (Nwankpa, 2008; Danbirni *et al*., 2010; Okaiyeto *et al*., 2011; Jasini *et al*., 2016). Therefore, data generated from this research can serve as the baseline data for future studies in the region.

Material and Methods

Study area

This study was undertaken in three selected South Eastern states of Nigeria (Anambra, Enugu and Imo states). The South-Eastern region is one of the geopolitical zones in the country. It consists of Anambra, Enugu, Imo, Ebonyi, and Abia states. Anambra state lies between latitude 5° 32′ and 6° 45′ N and longitude 6° 43′ and 7° 22′ E; Enugu state lies between latitude 5° 27′and 6° 33′ N and longitude 6° 28′ and 7° 32′ E, and Imo state is located between latitude 4° 45′ and 7° and 15′ N and longitude 6° 50′ and 7°25′ E. The region has an estimated cattle population of 4.5 million from a total of 16.3 million estimated cattle population in Nigeria (Ikhatua, 2011).

Sample collection and processing

A total of 90 bovine samples (65 lungs tissues and 25 pleural fluids) were collected from slaughtered animals showing classical signs of CBPP at the abattoir (Figs. 1 and 2). Samples were collected for 4 months between December 2019 and March 2020. The lung tissue samples were collected using sterile blades. Pleural fluids were collected using sterile 18G syringes (Fig. 1). Subsequently, all the collected samples were put in sterile sample bottles and transported in CBPP transport medium pleuropneumonia like organism (PPLO broth). They were properly labeled and conveyed to the Mycoplasma laboratory of NVRI, Vom for further processing.

Culture and isolation of Mycoplasma specie

The lung tissues and pleural fluids collected from the suspected cattle at the abattoir were cultured in PPLO growth medium adhering to specific protocols by the OIE manual (OIE, 2014). Briefly, the suspected lung tissue and pleural fluid were incubated in PPLO broth for at least 48 hours at 37°C under anaerobic condition. After the incubation period, 20 µl of the overnight broth was added to 180 µl of PPLO broth and a ten-fold serial dilution (10^{-1}–10^{-4}) was carried out and finally sub-cultured onto PPLO agar. The agar plates were incubated for at least 72 hours at 37°C in a medium having 5% CO_2 and were monitored for colony growth.

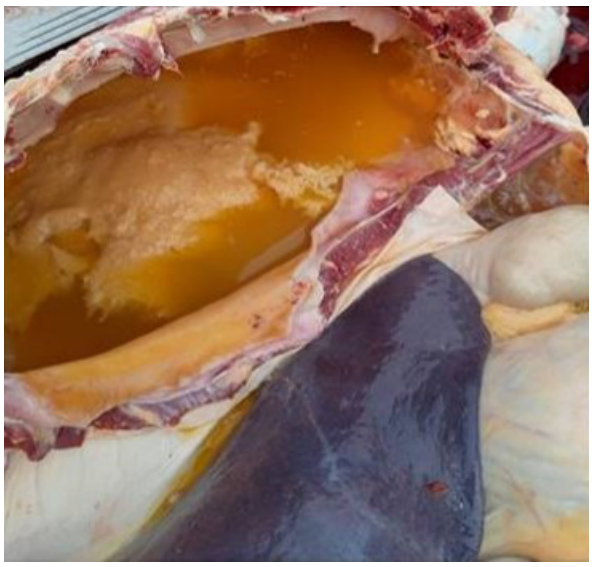

Fig. 1. CBPP infected animal at slaughter showing collection of pleural fluid.

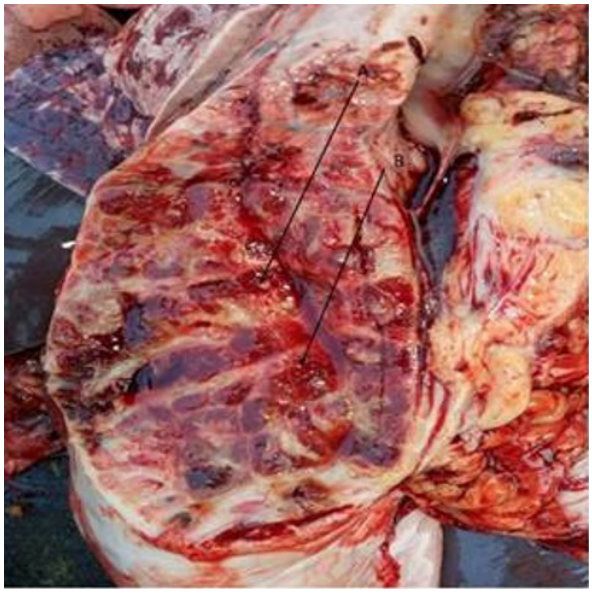

Fig. 2. Lung of CBPP infected cattle at post mortem showing thickening of the interlobular septa (marbled appearance) when cut open.

Small-to-medium sized colonies with the characteristic of a "fried egg" appearance under the stereomicroscope at 35× magnifications were considered presumptive of *Mmm*.

Confirmation of Mmm isolates using PCR

All the positive *Mycoplasma* isolates after culture on growth medium (PPLO agar) were subjected to conventional PCR assay for the detection of *Mmm* according to protocols by Miles *et al.* (2006).

Extraction of DNA

DNA was extracted from a 3 ml *Mmm* broth culture using QlAamp® DNA Mini kit. It was carried out according to the manufacturer's instructions. Lyophilized T1/44 vaccine (NVRI, Vom Nigeria) was used as the positive control for this study. Briefly, Proteinase K (20 µl) was pipetted into 1.5 ml micro-centrifuge tube after which 200 µl of the sample (broth culture) was added. Subsequently, 200 µl Buffer AL was then added and mixed thoroughly by vortexing for 15 seconds. This mixture was incubated at 56°C for 10 minutes. Thereafter, 200 µl ethanol was added and mixed thoroughly. The mixture was centrifuged at 6,000 × g for 1 minute. The resultant pellets were then washed twice in Buffer AW1 and AW2 respectively. Finally, 200 µl of Buffer AE was added and incubated at room temperature for 1 minute and centrifuged at 8,000 × g for 1 minute to elute the DNA. Thereafter, 2.5 µl of the DNA extract was used as the template for all the reactions.

Specific PCR protocol for the confirmation of M. mycoides subsp. mycoides

All the PCR reactions were performed in a total volume of 25 µl, which contained dH_2O, 5× FIREPol® master mix (12 mM MgCl, 1 mM dNTP mix, FIREPol® DNA polymerase and 1 µl IS1296F: Primer (5′–3′): CTA AAG AGC TTG GAG TTC AGT G and 1 µl R (all) (sequence 5′–3′): CCA GCT CAACCA GCT CCA G) (Miles *et al*., 2006).

DNA amplification was performed using GeneAmp® PCR system 2,720 (Perkin Elmer, Courtaboeuf, France). It consisted of an initial denaturation step at 95°C for 5 minutes, followed by 32 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute and 20 seconds. The final extension stage was maintained at 72°C for 5 minutes. The PCR product was then running on 2% agarose gel impregnated with ethidium bromide (0.5 µg/ml) at 130 volts for 30 minutes. Subsequently, the DNA migration was viewed under ultra-violent light and photographs were taken. The production of a band equivalent to 1.1 kbp and at the same distance with the positive control (T1/44) was considered confirmatory for *M. mycoides subsp mycoides.*

Ethical approval

The consent of the Ahmadu Bello University Animal Care and Use was sought after for this study. Samples were collected from already slaughtered animals at registered abattoirs. The abattoirs abided by the government established guidelines for slaughter.

Results

Culture and isolation of M. mycoides subsp. mycoides

Out of the 90 bovine samples (25 pleural fluids and 65 lung tissues) collected, 25 (27.7%) were considered positive for *M. mycoides* subsp. *mycoides* based on their colonial morphology on PPLO agar (Table 1). A typical fried egg colonies with some of the colonies having dense centre while others were raised with nipple like appearance were observed from day 3 of culture on PPLO agar (Figs. 3 and 4). A total of 30 samples (10 pleural fluid and 20 lung tissues) were collected from Enugu state of which 10 (33.3%) were positive on PPLO agar (Table 1). Similarly, 40 tissue samples (15 pleural fluid and 25 lung tissues) were collected from Anambra state of which 15 (37.5%) were positive on PPLO agar. There were no positive isolates from the 20 lung tissue samples from Imo state (Table 1).

Table 1. Number of *Mycoplasma mycoides* subsp. *mycoides* isolates positive on PPLO agar.

States	Number of samples	Number of positive on agar (%)
Anambra	40	15 (37.5%)
Enugu	30	10 (33.3%)
Imo	20	0 (0)
Total	90	25 (27.7%)

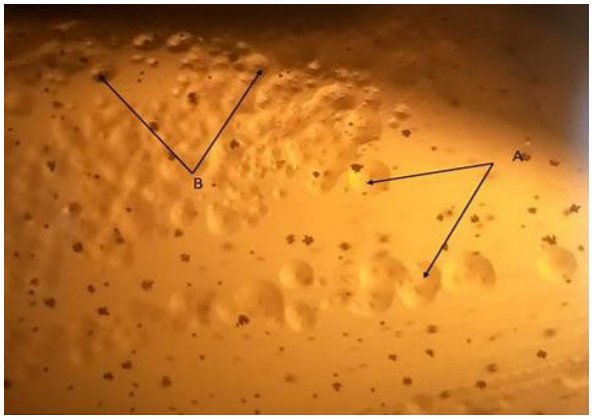

Fig. 3. *Mycoplasma mycoides* subsp. *mycoides* colonies on PPLO agar.

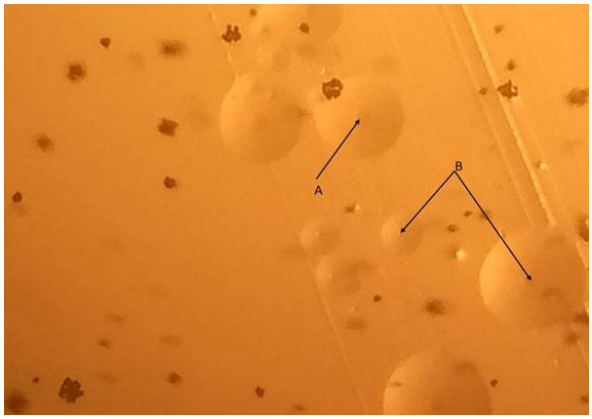

Fig. 4. *Mycoplasma mycoides* subsp. *mycoides* colonies (raised colonies with dark pinpoint centres) on PPLO agar.

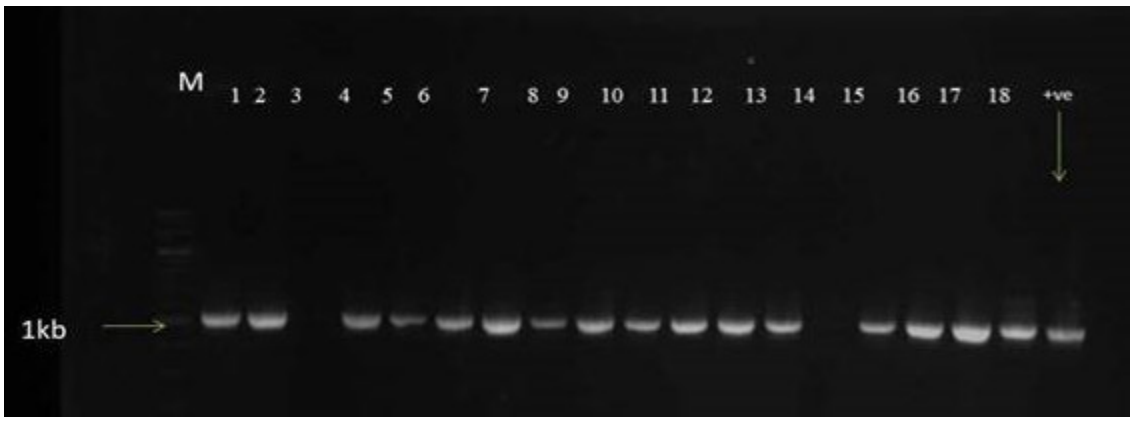

Fig. 5. *Mmm* specific PCR showing 1.1kb amplicon size. Lane M: 100 base pair ladder. Line 1-18: positive isolates. Lane +ve: for positive control.

Fig. 6. *Mmm* specific PCR showing 1.1kb amplicon size. Lane M: 100 base pair ladder. Lane 19-21: positive isolates. Lane +ve: for positive control.

Confirmation of Mmm by conventional PCR

Twenty one out of the 25 Mycoplasma isolates cultured on PPLO agar were confirmed as *M. mycoides* subsp. *mycoides* using conventional PCR as described by Miles *et al.* (2006). Production of a band equivalent to 1.1 kbp and at the same distance with T1/44 positive control confirms the presence of *Mmm* (Figs. 5 and 6).

Discussion

The South east region of Nigeria is estimated to have a cattle population of 4.5 million (Ikhatua, 2011). There is an apparent successful settlement of pastoralist within the Southern region of Nigeria in the past four decades with thousands of zebu cattle (Blench, 1994). However, most of the studies on CBPP are centered within the Northern region, where most of the cattle population is located.

In this study, the overall *Mmm* isolation rate was 27.75%. Twenty five of the isolates were positive on PPLO agar. This high isolation rate could be attributed to the fact that purposive sampling method was used for this study. Only apparently sick animals showing classical clinical signs and gross lesions suggestive of CBPP at the abattoirs were sampled. Samples such as pleural fluid and pneumonic lungs were collected from the abattoirs. Pleural fluid accumulation is one of the characteristic of CBPP and is considered as one of the best medium for the isolation of *M. mycoides* subsp. *mycoides* (Thiaucourt *et al*., 2006). Anambra state recorded the highest isolation rate of *Mmm*. Perhaps, more animals showing classical gross lesions of CBPP at the slaughter house were sampled in Anambra state followed by Enugu State. However, there was no isolate from Imo state. The failure to isolate *Mmm* in Imo state could be because most of the cattle sampled were apparently healthy, showing no classical gross lesions of CBPP at the abattoir. Similarly, the presence of antibiotics contamination in the collected samples may have also accounted for the inability to recover mycoplasma from the samples. The isolation rate in this

study is in contrast to studies by Jasini *et al.* (2016), who recorded an overall isolation rate of 3.33% in the North eastern region of Nigeria. Similarly, Nwankpa (2008), recorded 6.27% from cattle within Northern Nigeria. Ikpa *et al.* (2020) also recorded a lower isolation rate of 4% in Nasarawa State, Nigeria.

Several genomic and molecular tools have been developed for the identification and confirmation of *M. mycoides* subsp. *mycoides* (Bashiruddin *et al*., 1994: Miles *et al*., 2006). PCR is a robust technique for the detection, identification, and differentiation of members of the *M. mycoides* cluster (Miles *et al*., 2006). In this research, out of the total of 25 *Mmm* isolates positive on PPLO culture medium, 21 isolates were confirmed to be *M. mycoides* subsp. *mycoides* by PCR. The isolates yielded molecular size of 1.1 kbp specific for *Mmm*. This is in agreement with the findings by Miles *et al.* (2006). Generally, species within the *M. mycoides* cluster share many immunological, biochemical and genetic properties; which can result in major problems for diagnostic laboratories in the identification process of field strains (Cottew *et al*., 1987; Persson *et al*., 2002). Perhaps, some of the positive isolates on PPLO agar in this study were *Mycoplasma* species within the *Mycoplasma* cluster as the isolates were not subjected to biochemical tests. The use of PCR for the confirmation of *Mycoplasma* species from several clinical samples has shown a higher efficiency, specificity, and sensitivity for laboratory diagnosis when compared with conventional culture-based diagnostic procedures (Bashiruddin *et al*., 1994).

Conclusion

This study confirms the presence of *M. mycoides* subsp. *mycoides* in South eastern Nigeria. This is the first report on the isolation and confirmation of *Mmm* in South-eastern region of Nigeria. We, therefore, recommend active surveillance and vaccination programs within the region for the control and prevention of Contagious Bovine Pleuropneumonia.

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Conflict of interest

The authors declare no conflict of interest.

Authors contribution

SOO, CNK, and AKBS supervised the study. KCA, PDL, and PIA collected and analyzed the samples. KCA wrote the manuscript. All authors read and approved the manuscript for submission.

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