Identification of Mycoplasma mycoides *subspecies* mycoides *from slaughtered cattle in two transboundary states of North-eastern Nigeria*

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Summary
This study aimed to perform molecular typing of *Mycoplasma mycoides* subsp. *mycoides* from slaughtered cattle in Adamawa and Taraba States, north-eastern Nigeria. A total of four hundred and eighty (480) samples of lung tissues, nasal swabs, ear swabs and pleural fluids were collected from cattle at slaughter and processed according to standard laboratory protocols. Identification and confirmation were achieved with specific PCR and PCR-RFLP. An overall *M. mycoides* subsp. *mycoides* isolation rate of 6.87% (33/480) was obtained. In Adamawa State, 12 (10.91%) isolates of *M. mycoides* subsp. *mycoides* came from both, lung tissues and pleural fluids. While in Taraba State, 5 (7.14%) and 4 (5.71%) isolates of *M. mycoides* subsp. *mycoides* came from lung tissues and pleural fluids, respectively. The samples from nasal and ear swabs from the study states were negative for *M. mycoides* subsp. *mycoides*. Thirty-three out of the 37 culture positive isolates were confirmed to be *Mycoplasma mycoides* subspecies *mycoides* with the production of a band equivalent to 574-bp. Molecular typing with restriction endonuclease *Vsp*1 results in the two bands of 180-bp and 380-bp. In conclusion, the study has established an isolation rate of 6.87% for *M. mycoides* subsp. *mycoides*. Measures to strengthen movement control in order to minimise the spread of this dreaded disease of cattle were recommended.

Introduction

Mycoplasma mycoides subspecies *mycoides* (*M. mycoides* subsp. *mycoides*) is an important bacterium pathogen of cattle causing contagious bovine pleuropneumonia (CBPP), which is one of the most severe endemic infectious disease in sub-Saharan Africa. The disease is causing major social and economical impact, due to the animal losses and international trade restrictions (Mariner *et al.* 2006, Dupuy *et al.* 2012, Fischer *et al.* 2015, Parker *et al.* 2018). *M. mycoides* subsp. *mycoides* was the first *Mycoplasma* to be described, as extracellular bacterium that lives in close association with their host cells (Dupuy *et al.* 2012). The bacterium lacks of cell wall, and has the capacity of self-replication (Westberg *et al.* 2004, Di Teodoro *et al.* 2020).

Mycoplasma mycoides subsp. *mycoides* infects lung tissues and can be transmitted through inhalation of infectious aerosols of the organism by a susceptible animal (OIE 2018). Severe inflammatory, exudative lesions at lung and pleural membranes characterize the disease. In calves, however, infection of *M. mycoides* subsp. *mycoides* results mainly in arthritis and associated lameness, but rarely in pulmonary lesions (OIE 2015). Other factors such as movement of trade cattle, seasonal migration, transhumance and normadism also enhance the spread of the infection (Aliyu *et al.* 2000).

The WOAH recommended complement fixation test (CFT), competitive enzyme-linked immunosorbent assay (c-ELISA) and polymerase chain reaction for the diagnosis of CBPP (OIE 2018). Isolation

and identification of mycoplasmas is still the gold standard even though it requires a well-equipped laboratory with expertise for these very fastidious and slow-growing organisms (McAuliffe *et al.* 2005, Egwu *et al.* 2012). The use of PCR for the rapid and specific identification of *M. mycoides* subsp. *mycoides* has its limitations in developing countries, but detection of these species by PCR proved to be highly efficient with better results than culturing techniques (Egwu *et al.* 2012, Wade *et al.* 2015).

The disease threatens livestock production, limits international trade and is therefore of huge economic concern in affected countries (Fadiga *et al.* 2013). In Nigeria, estimated CBPP morbidity and mortality rate of up to 50% and 25%, respectively, have been documented with annual economic losses of more than 2.2 billion Nigerian Naira (Fadiga *et al.* 2013). Outbreaks of the disease still occur in the northern region which harbours three-quarter of the country's 19.5 million cattle population (Tambuwal *et al.* 2011).

Several epidemiological studies have been conducted in Nigeria to assess the situation of CBPP in the North-eastern region (Ameh *et al.* 1998, Aliyu *et al.* 2000, Francis *et al.* 2018a) but there was no report on the molecular typing of the causative agent. Recent information about the disease is scanty and no extensive work been done in the study area. The present study was aimed to perform molecular identification and typing of *M. mycoides* subsp. *mycoides* from slaughtered cattle in Adamawa and Taraba States, North-eastern Nigeria, with the aim to help in clarifying the characteristics of the strains circulating in the study area.

Materials and methods

Study area

Adamawa and Taraba States are located in the South-eastern part of Nigeria (Figure 1). They lie between latitude 7°0′0″ and 11°0′0″N and between Longitude 9°0′0″ and 13°0′0″E. The Yola Modern Abattoir in Adamawa State is located on the Latitude 9°13′33.3″N and Longitude 12°27′10.1″E. An average of 70-80 cattle is slaughtered daily in the abattoir, while numbers are higher during festivity period. Jalingo Abattoir in Taraba State is located on the Latitude 8°54′12.1″N and Longitude 11°21′14.3″E with an average of 40-50 cattle slaughtered daily. Adamawa State shares boundaries with Taraba, Borno, Gombe States while Taraba State is bounded by Gombe, Bauchi, Plateau, Nassarawa and Benue States. They both share an international boundary with the Cameroon Republic along the southeastern border. Adamawa and Taraba States have a land area of about 91,390 km^2 with tropical wet and dry climate. The two states are among the lead producers of livestock in Nigeria with an estimated cattle population of 3.5 million (Francis *et al.* 2018b).

Sample collection and storage

The samples used for this study were obtained from cattle slaughtered at abattoirs within the areas under study between 2016 and 2017. The samples were collected for a period of thirteen (13) months. A total of four hundred and eighty (480) samples of pneumonic suspected lung tissues (180), nasal swabs (180), ear swabs (60) and pleural fluid (60) were collected from 190 cattle heads at slaughter, when they were showing pathological lesions suspected for CBPP. Two hundred and eighty four (284) and one hundred and ninety six (196) samples from cattle were collected from the abattoirs in Adamawa and Taraba States, respectively (Table I). All the collected samples from abattoirs in Yola and Jalingo were transported to the National Veterinary Research Institute Zonal Offices in Yola and Jalingo, respectively. Samples were processed in 2 ml sterile Nalgene® cryo vials containing 1.5 ml of pleuropneumonia-like organism (PPLO) broth (Difco) supplemented (horse serum, sodium pyruvate and fresh yeast extract) and were later transported in refrigerated Coleman box to the *Mycoplasma* Laboratory, National Veterinary Research Institute (NVRI) Vom, Plateau State and then stored at - 20 °C prior to further processing.

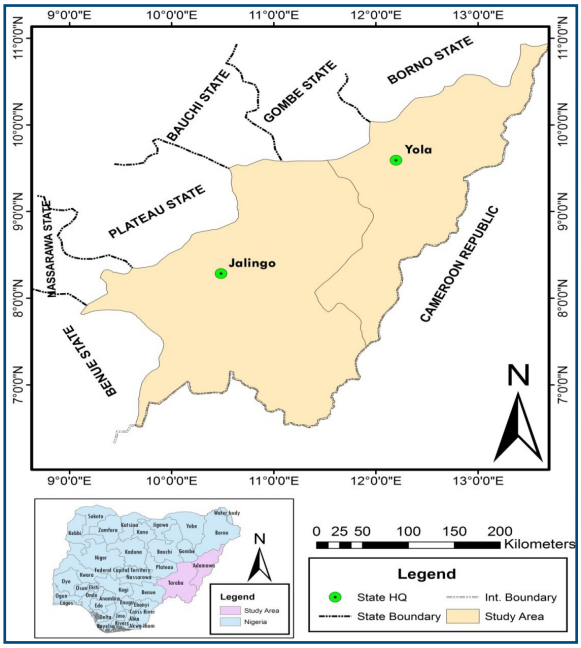

Figure 1. *Map of Adamawa and Taraba State, North-eastern Nigeria showing the study areas.*

Isolation of mycoplasmas

The samples were processed according to the method described by Thiaucourt and colleagues (Thiaucourt *et al.* 1992). The PPLO broth (Difco) supplemented, containing various samples were filtered by passing through 0.45 µm millipore filters (Millipore Merck, Burlington, MA, USA) which is permeable to organisms smaller than 0.45 µm in size. The filtrate was then incubated at 37 °C in screw-capped bottles containing supplemented PPLO broth (Difco) for three (3) days under 5% CO_2 and later cultured onto PPLO agar (Difco) supplemented. These were incubated at 37 °C in a 5% CO_2 atmosphere with maximum humidity, and examined daily for a period of 1-2 weeks for evidence of growth, using a stereomicroscope. Isolated colonies having the classical 'fried eggs' appearance with dense raised centres (nipples) were triple-cloned into 10 ml of PPLO broth (Difco), and incubated for one week. Both, broth and agar media were considered negative if no comet or *Mycoplasma*-like colonies were observed after 14 days of incubation. Cloned isolates were stored at + 4 °C. These isolates were later transported refrigerated at 2-8 °C by Express Courier to the WOAH Reference Laboratory for CBPP at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' (IZSAM), Teramo, Italy. Upon receipt, the isolates were stored at + 4 °C, verified and then registered under the exotic disease unit before being processed.

Identification and confirmation of *Mycoplasma mycoides* subsp. *mycoides* isolates

DNA extraction

Deoxyribonucleic acid was extracted from 500 µl of PPLO broth presenting comet colonies using Maxwell® 16 Tissue/cell DNA Purification kits (Promega, Wincosin, USA) with Maxwell® 16 instrument (Promega, Wincosin, USA) according to the manufacturer's instructions and the extracted DNA was kept at - 20 °C until analyzed.

Identification of Mycoplasma *species using specific PCR*

The nucleic acid from each sample was tested using a PCR specific for *Mycoplasma* species as previously described (van Kuppeveld *et al.* 1994). The *Mycoplasma* group-specific primer set which amplifies a 280-bp fragment, consists of the forward primer 5'-GGGAGCAAACAGGATTAGATACCCT-3' and the reverse primer 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'. The aliquots were analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide (van Kuppeveld *et al.* 1994). This PCR was able to detect *Mycoplasma* spp. in general and it is routinely used to confirm the presence of mycoplasmas in samples. Isolates that yielded positive to *Mycoplasma* species underwent additional testing using a *Mycoplasma* specific PCR.

Identification of Mycoplasma mycoides *subspecies using PCR*

This protocol is a PCR-based test for the specific identification of *M. mycoides* subspecies according to Bashiruddin and colleagues (Bashiruddin *et al.* 1994). For molecular characterization, the nucleic acid of the samples were further amplified using a set of primers: MM450 (forward) 5'-GTATTTTCCTTTCTAATTTG-3' and MM451 (reverse): 5'-AAATCAAATTAAGTTTG-3' targeting the CAP-21 genomic region of 16S rRNA. An initial denaturation at 94 °C for 5 minutes was followed by 40 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 30 seconds, and then a final extension at 72 °C for 5 minutes. *Mycoplasma mycoides* sub-clusters were used as positive controls and the expected positive PCR products of 574 bp were visualized on a 1% agarose (Musa *et al.* 2016).

The PCR products were electrophoresed on 1% agarose gel and the samples run for 30 minutes. The resultant bands were stained with ethidium bromide and visualised under ultraviolet light (Bashiruddin *et al.* 1994).

Differentiation of Mycoplasma mycoides *subspecies* mycoides *using PCR-RFLP*

The typing method was adapted from the protocol used in WOAH Reference Laboratory for CBPP and that of Bashiruddin and colleagues (Bashiruddin *et al.* 2005), but varied with respect to *Vsp*1 (8-12 U/µl concentration) which replaced *Asn*1 endonuclease in this study. The PCR-RFLP was performed according to the method described by Musa and colleagues (Musa *et al.* 2016).

Results

Out of the 480 samples tested, 39 (8.13%) were positive for *Mycoplasma* species by culture. In Adamawa State, 25 (8.80%) isolations were made from 12 (10.91%) lung tissues and 13 (11.82%) pleural fluid. In Taraba State, 8 (11.43%) lung tissues, 5 (7.14%) pleural fluid and 1 (3.57%) ear swab yielded a characteristic 'fried egg' colonies, typical of *Mycoplasma*, with an isolation rate of 7.14% (n = 14).

The thirty nine (39) pure isolates of *Mycoplasma*

species were shipped to the above mentioned WOAH Reference Laboratory for CBPP, where 37 isolates were confirmed on both PPLO media and PCR specific for *Mycoplasma* species. Thus, 19 (10.56%) lung tissues, 17 (9.44%) pleural fluid and 1 (1.67%) ear swab yielded positive results, leading to an overall *Mycoplasma* species recovery rate of 7.70% (37/480), out of which Adamawa State had 25 (8.80%) and Taraba States had 12 (6.12%). More isolates came from Adamawa State compared to Taraba State. The isolation and recovery rates of *Mycoplasma* species are shown on Table I.

Mycoplasma mycoides subsp. *mycoides* (Figure 2) was the dominant species 33 (6.87%). In Adamawa State, 12 (10.91%) isolates of *M. mycoides* subsp. *mycoides* came from both, lung tissues and pleural fluids. The study revealed that in Taraba State 5 (7.14%) and 4 (5.71%) isolates of *M. mycoides* subsp. *mycoides* came from lung tissues and pleural fluid, respectively. The samples from nasal and ear swab from the study area were negative for *M. mycoides* subsp. *mycoides* (Table I).

Thirty-three (33) out of the thirty-seven (37) *Mycoplasma* isolates were positive for PCR, showing a band of 574-bp (Figure 3). All the PCR positive samples were characterized as *M. mycoides* subsp. *mycoides* by PCR-RFLP method (Figure 4).

Discussion

The isolation rate of 6.87% of *M. mycoides* subsp. *mycoides* found in this study was slightly higher than the previous investigations carried out in Northern and Central Nigeria (Ankeli *et al.* 2016, Musa *et al.* 2016, Tambuwal and Egwu 2017). The increased rate observed in the same agro-ecological zone may be connected to the social security situation in the North-eastern Nigeria, where there are fragmented veterinary services due to insurgency (Aliyu

Table I. *Isolation rates of* Mycoplasma *species and identification of* M. mycoides *subsp.* mycoides *(*Mmm*) from cattle in Adamawa and Taraba States, Nigeria.*

Source of sample	Sample type	No. of samples collected	No. of *Mycoplasma* cultured (%)	No. of *Mycoplasma* confirmed (%)	No. of *Mmm* identified (%)
Adamawa State	Lungs	110	12 (10.91)	12 (10.91)	12 (10.91)
	Pleural fluid	110	13 (11.82)	13 (11.82)	12 (10.91)
	Nasal swab	32	0 (0.00)	0 (0.00)	0 (0.00)
	Ear swab	32	0 (0.00)	0 (0.00)	0 (0.00)
Sub-total		284	25 (8.80)	25 (8.80)	24 (8.45)
Taraba State	Lungs	70	8 (11.43)	7 (10.00)	5 (7.14)
	Pleural fluid	70	5 (7.14)	4 (5.71)	4 (5.71)
	Nasal swab	28	0 (0.00)	0 (0.00)	0 (0.00)
	Ear swab	28	1 (3.57)	1 (3.57)	0 (0.00)
Sub-total		196	14 (7.14)	12 (6.12)	9 (4.59)
TOTAL		480	39 (8.13)	37 (7.71)	33 (6.87)

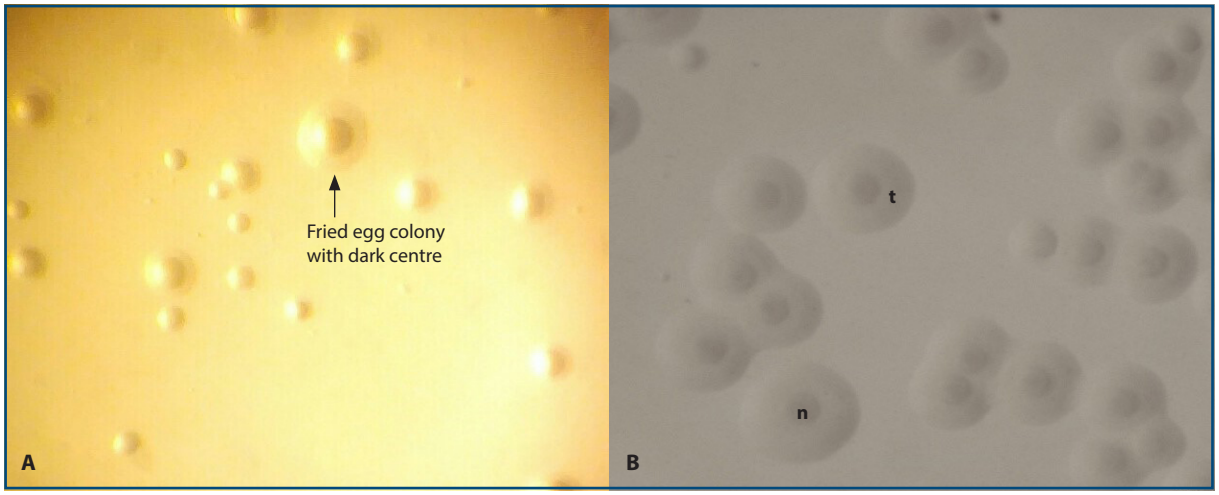

Figure 2. *Colonies of* M. mycoides *subspecies* mycoides *from lung sample on PPLO agar showing characteristic 'fried egg shape' colonies, dense raised central area 'nipple' (n) and transparent periphery (t) growth on PPLO agar isolated at NVRI Vom after 72 hours (***A***) and recovered from isolates shipped to IZSAM Teramo, Italy after 5 days (***B***) of incubation viewed under stereomicrospe (X35).*

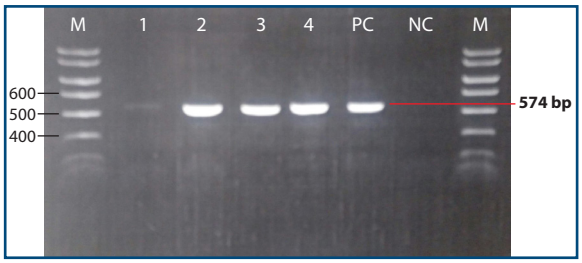

Figure 3. *PCR amplification of* M. mycoides *subspecies CAP-21 genomic region (574-bp) on 1% agarose gel for identification using primers MM450 and MM451.* Lane M = Molecular size marker 100-bp; Lanes 1-4 = Samples showing *Mm* subspecies; PC = Positive control; NC = Negative control.

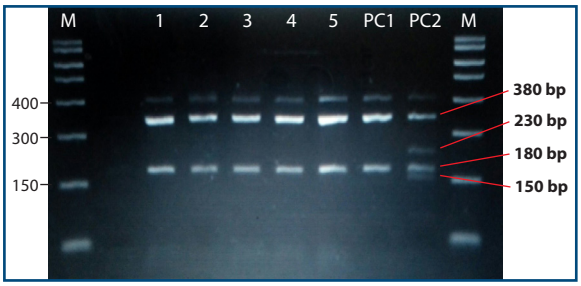

Figure 4. *PCR-RFLP amplicons on 3% agarose gel for the identification of* M. mycoides *subspecies* mycoides *following digestion of PCR product with restriction endonuclease* Vsp*1.* Lane M = Molecular size marker 100-bp; Lanes 1-5 = Positive samples showing *M. mycoides* subsp. *mycoides* profile; lane PC1 = *M. mycoides* subsp. *mycoides* positive control; lane PC2 = *Mm* subsp. *capri* positive control.

et al. 2000, Francis *et al.* 2018a). To the best of our knowledge, this is the highest reported isolation rate of *M. mycoides* subsp. *mycoides* from field samples in Nigeria. The high isolation rate of *M. mycoides* subsp. *mycoides* obtained in this study may indicate that the disease is very active and endemic in the study area. The increased and unrestricted cattle movements, as well as the porous nature of borders with neighbouring countries, might have among other factors contribute to the high rate observed in this study (Aliyu *et al.* 2000).

Mycoplasma mycoides subspecies *mycoides* was not isolated from the nasal and ear swab samples collected, even though WOAH recommended these sites for sampling in live animals (Karahan *et al.* 2010, OIE 2015). This may be due to contamination of such sites with debris, ear mites, other bacterial and *Mycoplasma* flora, thus suppressing *M. mycoides* subsp. *mycoides* from thriving in such anatomical sites (Santos *et al.* 2009). Compared to nasal and ear swabs, the broncho-alveolar lavage (BAL) method of sampling is the most preferred method for isolation of *Mycoplasma* in live animals, to avoid sample contamination. However, the BAL sampling method was much more difficult to perform under field conditions, and animal owners are usually reluctant to allow this procedure (Karahan *et al.* 2010, Akan *et al.* 2014). The results obtained in the present study are in contrast with the previous report of Ankeli and colleagues (Ankeli *et al.* 2016), who reported isolation of *M. mycoides* subsp. *mycoides* from the ear canal of apparently healthy cattle in Plateau State.

Molecular typing of thirty-three isolates out of the original thirty-seven was quite significant and showed that CBPP caused by *M. mycoides* subsp. *mycoides* was active, endemic and widespread in the study area. This finding is in agreement with previous reports (Bashiruddin *et al.* 2005, Muuka *et al.* 2013, Wade *et al.* 2015, Musa *et al.* 2016). The digested restriction endonuclease 380-bp fragments delineated *M. mycoides* subsp. *mycoides* from *M. mycoides* subspecies capri. This finding corroborates the earlier reports (Bashiruddin *et al.* 1999, Musa *et al.* 2016) and it further revealed strains of *M. mycoides* subsp. *mycoides* circulating in Adamawa and Taraba States, Nigeria.

In conclusion, the present study has established the occurrence of *M. mycoides* subsp. *mycoides* North-eastern part of Nigeria. Also, a high number of *M. mycoides* subsp. *mycoides* field strains have been isolated which is to the best of our knowledge the first figure of this kind in Nigeria. It is recommended to strenghten animal movements control at Nigerian national and international borders, in order to minimise the spread of this dreaded and internationally recognised disease of cattle.

Statement of animal rights

As at the time of kick-starting this study, Ahmadu Bello University Zaria, Nigeria did not have any committee mandated to authorise research works. Though, the research procedure employed in this study was presented to the Postgraduate Board of Faculty of Veterinary Medicine, which considered ethical and welfare standards before approval was given.

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