Research Article

Isolation and Identification of *Mycoplasma mycoides* Subspecies *mycoides* from the Ear Canal of Cattle in Plateau State, North-Central Nigeria

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Abstract | This study was undertaken to isolate and identify *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) from the ear canal of apparently healthy cattle in Plateau State, Nigeria. One hundred and sixty six ear swab samples (n=166) were cultured from which eight (8) *Mycoplasma* species were isolated and characterized using conventional biochemical tests and polymerase chain reaction (PCR), respectively. Six (6) were observed to ferment glucose, reduce tetrazolium chloride and hydrolysed casein. They had no phosphatase activity, did not produce 'film and spots' and neither hydrolysed arginine nor urea and were preliminarily identified as members of the *Mycoplasma mycoides* sub-cluster which comprises *Mmm* and *Mycoplasma mycoides* subspecies *capri* (*Mmc*). Three (3) of the 8 *Mycoplasma* isolates were identified as *Mmm* using the IS1296F/R(all) and MM450/MM451 primer sets. The 574 bp product obtained by MM450/MM451 primer set gave two fragments (379, 178 bp) on digestion with *Asn*I restriction enzyme further confirming the isolates. This is the first report of the isolation and molecular identification of *Mmm* from the ear canal of cattle in Nigeria. It is recommended that samples from the ear canal be considered when *Mmm* isolation is intended so as to improve the chance of recovery.

Editor | Muhammad Abubakar, National Veterinary Laboratories, Park Road, Islamabad, Pakistan.
Received | June 08, 2016; **Accepted** | July 22, 2016; **Published** | August 29, 2016
***Correspondence** | Paul I. Ankeli, Bacterial Vaccine Production Division, National Veterinary Research Institute, Vom, Plateau State, Nigeria; **Email:** drpaulidokoankeli@yahoo.com
Citation | Ankeli, P.I., O. Fagbamila, L.T. Ikpa, I.A. Muraina, P.D. Luka, M.A. Raji, H.M. Kazeem, M.O. Odugbo, N.J. Umaru, I.O.O. Nwankiti and N.D. Nwankpa. 2016. Isolation and identification of *Mycoplasma mycoides* subspecies *mycoides* from the ear canal of cattle in Plateau State, North-Central Nigeria. *Veterinary Sciences: Research and Reviews*, 2(2): 52-59.
DOI | http://dx.doi.org/10.17582/journal.vsrr/2016.2.2.52.59

Introduction

Mycoplasma mycoides subspecies *mycoides* (*Mmm*), the causative agent of contagious bovine pleuropneumonia (CBPP) in cattle, belongs to the genus *Mycoplasma* in the class *Mollicutes* (Westberg et al., 2004; Pilo et al., 2007; Li et al., 2009). It was the first mycoplasma to be described (Nocard and Roux, 1898) and has been studied extensively ever since (Tardy et al., 2011). Phylogenetically, *Mmm* is a member of the *Mycoplasma mycoides* cluster which are pathogens of ruminants that include *M. mycoides* subsp. *capri* (*Mmc*), *M. capricolum* subsp. *capripneumoniae* (*Mccp*), *M. capricolum* subsp. *capricolum* (*Mcc*) and *Mycoplasma leachii* (Manso-Silvan et al., 2009). These mycoplasmas are difficult to differentiate because of their genotypic and phenotypic relatedness (Tardy et al., 2009). However, the precise identification of *Mmm* is critical as it causes CBPP which is currently regarded as the most important cattle

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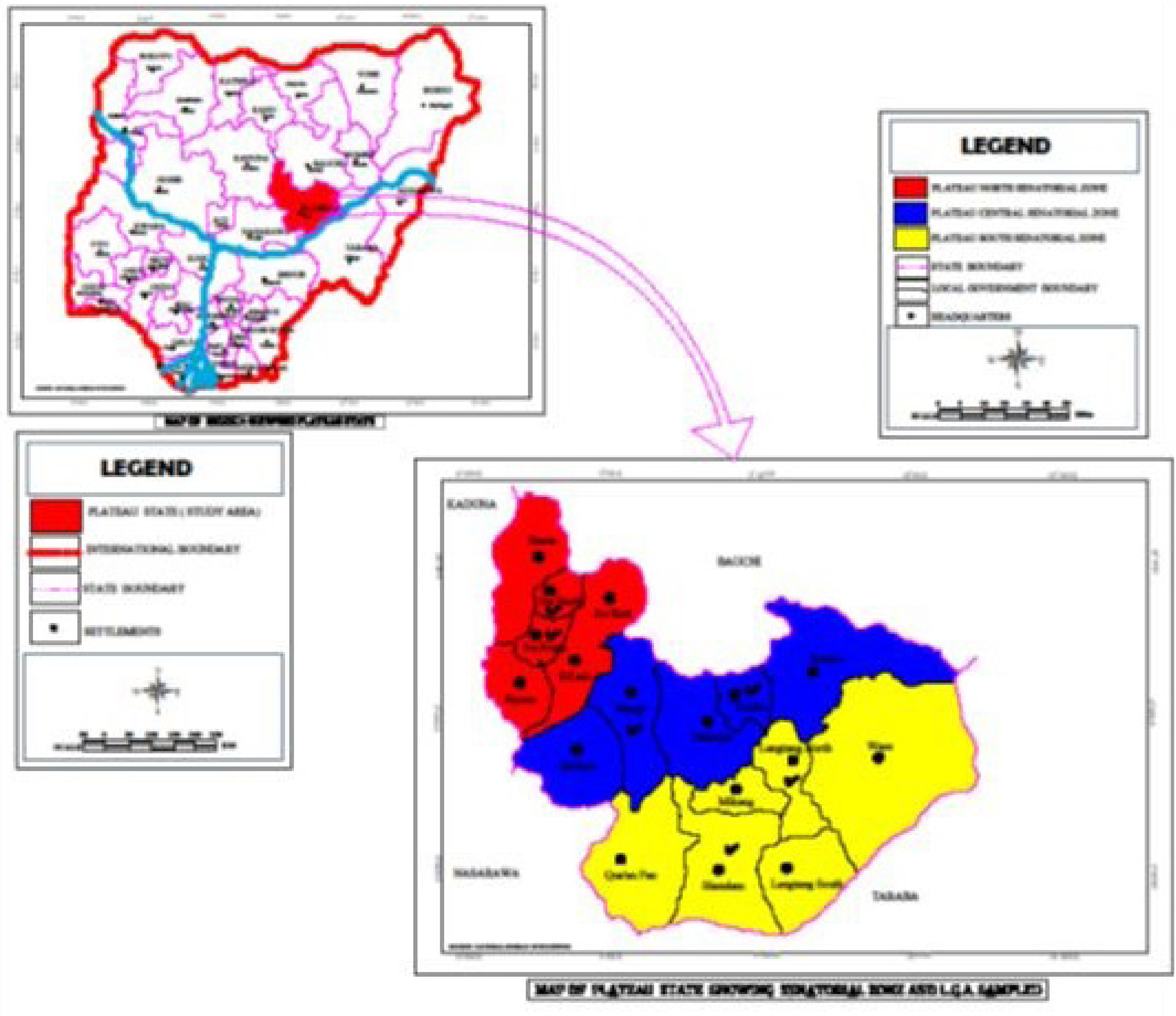

Figure 1: *Map of Plateau State showing Local Government Areas sampled (Source: National Bureau of Statistics)*

disease in sub-Saharan Africa since the eradication of rinderpest (Thiaucourt et al., 2000; Gull et al., 2013).

Several genomic techniques have been developed which enable the identification of *Mycoplasma* species (McAuliffe et al., 2005; Stakenborg et al., 2005) and robust diagnostic systems based on polymerase chain reaction (PCR) are available for the rapid detection, identification and differentiation of members of the *Mycoplasma mycoides* cluster and the specific identification of *Mmm* (Nicholas et al., 2008). Polymerase chain reaction (PCR) has enhanced a better resolution in the diagnosis and differentiation of mycoplasmas compared to conventional methodologies of culture and serology (Woubit, 2008) and thereby made detection and identification of *Mmm* more sensitive and reliable (Bashiruddin et al., 1994; Miles et al., 2006).

Isolation, though labour intensive and limited in sensitivity, remains the gold standard for the diagnosis of mycoplasma infections (Egwu et al., 2012; OIE, 2014). Several *Mycoplasma* species have been isolated from the ear canal of cattle some of which include *M. bovis* (Maeda et al., 2003; Francoz et al., 2004; Foster et al., 2008; Santos et al., 2012), *M. alkalescens* (Lamm et al., 2004; Nwankpa, 2008; Santos et al., 2012), *M. arginini*, *M. bovirhinis*, *M. capricolum*, *M. conjunctivae*, *M. mycoides* subsp. *capri* (Santos et al., 2012) and *M. leachii* (Nwankpa, 2008) but from available literature, *Mmm* isolation from the ear canal of cattle has not been documented. The ear canal is an unusual anatomical site for *Mmm* recovery, thus the aim of our study was to isolate and identify *Mmm* from the ear canal of apparently healthy cattle in Plateau State, North Central Nigeria (Figure 1).

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Materials and Methods

Study Area

Plateau State is located in the Middle Belt zone of Nigeria and lies between Latitudes 8° and 10° North and Longitudes 7° and 11° east. It shares common boundaries with four of the thirty six states of the Federation. Though situated in the tropical zone, the climate of the State is the nearest equivalent of temperate climate with mean temperatures of 18°C and maximum of 30°C, while mean annual rainfall varies between 1317.5 mm in the Southern part and 1460.0 mm on the high Plateau. Cattle are the major source of meat supply in Plateau State.

Sample Collection and Processing

A total of 166 swab samples of the ear canal were collected from live cattle in 6 markets (n = 106) and 12 farms (n = 60) respectively, from May 2013 – March 2016. The swabs were cut directly into Bijou bottles containing 3 ml mycoplasma broth culture medium (used as transport medium) and screwed tight. The mycoplasma broth medium contained 21g/l PPLO broth powder (Difco®), 20% de-complemented horse serum (Quad Five®), 10% fresh yeast extract, 0.2% glucose (Fluka®), 0.4% sodium pyruvate (MRS Scientific®) and 0.04% ampicillin (Quadrant®), was prepared according to the methods described by Freundt (1983) and Provost et al. (1987). Samples were kept on ice and transported to the Mycoplasma Laboratory, National Veterinary Research Institute, Vom, Plateau State, Nigeria for processing.

Cultivation and Preliminary Identification of *Mycoplasma* Species

Ear swab samples in transport medium (mycoplasma broth) were processed for mycoplasma isolation as described by the OIE manual (2014). Briefly, a four ten-fold serial dilution (10^{-1} to 10^{-4}) in mycoplasma broth medium was carried out. A 100 µl of the last dilution (10^{-4}) was inoculated into Bijou bottle containing 1.9 ml of mycoplasma broth medium and incubated at 37°C in aerobic conditions for 3 days later and sub-cultured onto mycoplasma agar plates. Agar plates were incubated at 37°C in 5% carbon dioxide under maximum humidity and monitored for evidence of mycoplasma-like ("fried egg") colonies every 3 days for a period of 2 weeks using a stereomicroscope at x35 magnification. Isolated colonies having classical 'fried egg' appearance with dense centers were triple-cloned, mixed with equal volume of glycerol (used as a cryo-preservative) and stored in duplicates at -20°C and -80°C, respectively.

For the preliminary identification of the isolates; the growth on mycoplasma media without serum as well as without antibiotic, and the classical biochemical tests which include glucose fermentation, arginine hydrolysis, tetrazolium reduction, phosphatase production, and urea and casein hydrolysis as well as production of 'film and spots' were carried out on each of the isolates as described by Poveda (1998).

Genome Amplification of *Mmm* isolates

DNA was extracted from isolates using ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research Corp., Irvine, CA, USA) according to manufacturer's instructions. Lyophilized T1/44 vaccine (NVRI, Vom Nigeria) was used as control for this study. The presence of *Mmm* DNA was first diagnostically confirmed by the amplification of 1.1 kbp and 574 bp fragments using the protocol described by Miles et al. (2006) and Bashiruddin et al. (1994), respectively. The 574 bp PCR products were further digested by *Asn*I as described by Bashiruddin et al. (1994) to obtain two fragments (379, 178 bp) for *Mmm* and three fragments (220, 178, 153 bp) for *Mmc*, respectively. PCR products obtained were resolved on 1.0% agarose gel impregnated with ethidium bromide (0.5ug/ml) at 130 volts for 50 minutes and visualized on Bio Rad Gel Doc™ XR+ (BioRad, CA, USA) system.

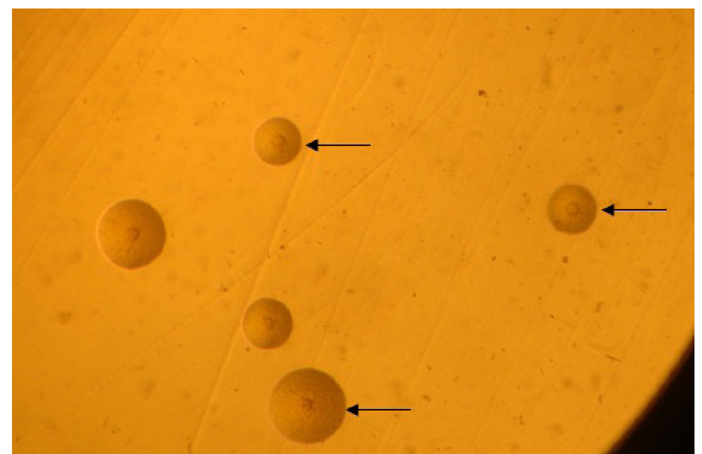

Figure 2: *Photomicrograph of Mycoplasma mycoides subspecies mycoides (Mmm) colonies isolated from ear swab samples from cattle on mycoplasma agar. Note: raised dense centres and lighter peripheries (arrows), x35*

Results

Mycoplasma Isolation

A total of 166 ear swab samples collected for *Mmm*

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Table 1: *Biochemical characteristics of mycoplasmas isolated from the ear canal of cattle in Plateau State*

	Biochemical tests							
Number of isolates recovered (%)	GF	AH	TR	PP	UH	CH	FS	Remarks
6 (75.0%)	+	-	+	-	-	+	-	*Mmm/Mmc*
2 (25.0%)	-	+	-	+	-	-	-	*M. alkalescens*

GF: *glucose fermentation;* **AH**: *arginine hydrolysis;* **TR**: *tetrazolium reduction;* **PP**: *phosphatase production;* **UH**: *urea hydrolysis;* **CH**: *casein hydrolysis;* **FS**: *film and spot production*

isolation, 8 yielded mycoplasma-like isolates when cultured on mycoplasma media. Mycoplasma-like growth observed in broth varied from a homogeneous cloudiness to slight turbidity, produced within 3–4 days, occasionally with a silky, fragile filament when broth media bottles were swirled. The growth observed on mycoplasma agar were characteristically small, circular, and convex with raised dark centres and lighter peripheries giving a 'fried-egg' appearance (Figure 2) as described by the OIE Manual (2014).

Preliminary Identification of the *Mycoplasma* Isolates

Growth of Mycoplasma isolates in serum-free and in antibiotic-free mycoplasma media. None of the isolates grew in serum-free mycoplasma medium (sterol dependency test) and all maintained the 'fried egg' appearance of colonies on mycoplasma agar medium without antibiotic, after 3 passages. They were thus designated mycoplasmas belonging to the family *Mycoplasmataceae* (*Mycoplasma* or *Ureaplasma* species), ruling out *Acholeplasma* species and L-form bacteria respectively, and giving an overall *Mycoplasma* species isolation rate of 4.82% from the ear canal.

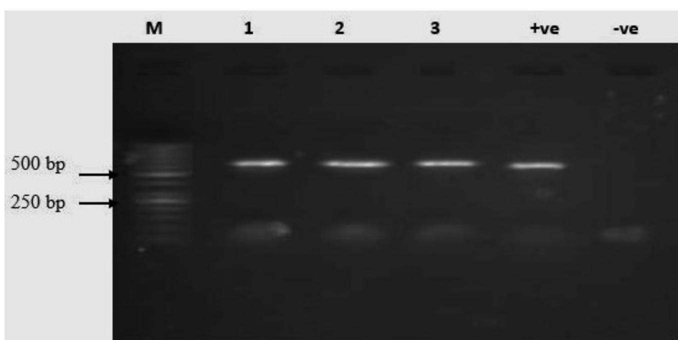

Figure 3: *Mmm-specific PCR showing 574 bp: Lane M is the molecular marker (50 bp ladder), Lane 1, 2, 3, for the three positive Mmm, respectively, Lane +ve, –ve for negative and positive (T1/44 vaccine strain) controls*

Biochemical Characterization of *Mycoplasma* Species

Biochemical characterization of the 8 mycoplasma isolates indicated that 6 fermented glucose, reduced tetrazolium chloride and hydrolysed casein. They did not hydrolyze arginine nor urea and neither produced phosphatase and 'film and spots', and were presumptively identified as members of the *Mycoplasma mycoides* sub-cluster (either *Mmm* or *Mmc*). Both *Mycoplasma* subspecies share these biochemical properties even though *Mmc* is a pathogen mostly of small ruminants. The other 2 mycoplasma isolates only hydrolysed arginine and produced phosphatase and were presumptively identified as *M. alkalescens*. *Ureaplasma* and other urea-utilizing *Mycoplasma* species were ruled out as none of the mycoplasma isolates hydrolysed urea (Table 1).

Molecular Identification of *Mmm* using PCR

A total of 8 mycoplasma isolates were subjected to PCR for identification. Out of them, 6 isolates presumptively identified by biochemical characterization as members of the *Mycoplasma mycoides* sub-cluster (*Mmm* and *Mmc*), 3 were positively identified as *Mmm* by PCR specific primers as described by Miles et al. (2006). The 6 presumptive *Mycoplasma mycoides* sub-cluster members gave 574 bp (Figure 3) following amplification by M450/M451 (Bashiruddin et al., 1994). Out of 6, three each were identified as *Mmm* and *Mmc* by producing two and three fragments each with the *AsnI* restriction enzyme (Bashiruddin et al., 1994). The remaining 2 mycoplasma isolates that hydrolysed arginine and produced phosphatase were negative for both *Mmm* and *Mmc* specific PCR and were therefore excluded from the study (Figure 4; Table 2).

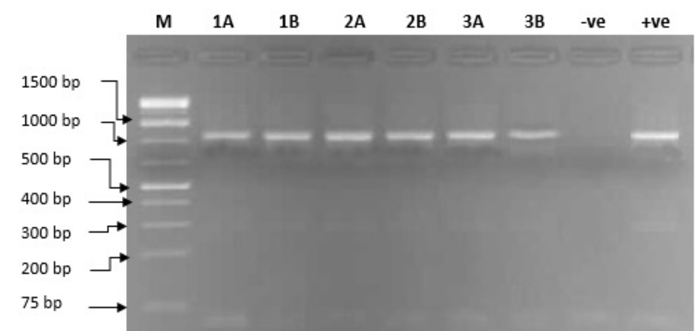

Figure 4: *Mmm-specific PCR showing 1.1 kb amplicon sizes ran in duplicates: Lane M is the molecular marker (1kp plus ladder), Lane 1A-1B, 2A-2B, 3A-3B, for the three positives, respectively, Lane –ve, +ve for negative and positive (T1/44 vaccine strain) controls*

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Table 2: *Isolation rate of Mmm from ear canal of cattle in Plateau State*

Sample type	Number examined	Number positive	Isolation rate (%)
Cattle markets	106	2	1.89
Cattle farms	60	1	1.67
Total	**166**	**3**	**1.81**

Discussion

The ear canal has now been included as one of the recommended sampling sites for pathogenic mycoplasmas in small ruminants (DaMassa and Brooks, 1991; Nicholas and Baker, 1998) and cattle (Santos et al., 2012). In view of the well-documented recoveries of mycoplasmas other than *Mmm* from the ear canal of cattle, its presence in the ear canal is therefore not unlikely. However, information on the isolation of *Mmm* from the ear canal of cattle is not available in literature. This might be attributed to the fact that previous studies were carried out in CBPP-free regions of the world (Chazel et al., 2010; Tardy et al., 2011; Santos et al., 2012), or due to the non-inclusion of culture for *Mycoplasma* species in the investigation of most auricular diseases (Vestweber, 1999; Francoz, 2004). Alternatively, the dearth of information on the isolation of *Mmm* from the ear canal of cattle can also be as a result of the collection of too few ear canal samples for *Mycoplasma* species isolation considering that the predilection site for most mycoplasmas is the respiratory system (Thiaucourt et al., 2004); other than *Mmm* from the ear canal.

Considering the ability of other ruminant mycoplasmas to colonise the ear canal, ear involvement by *Mmm* is not unlikely. Several hypotheses can be suggested to explain the occurrence of *Mmm* in the ear canal. Firstly, its extension from the pharynx via the Eustachian tube to the middle ear as described by Walz et al. (1997), Maeda et al. (2003) and Lamm et al. (2004) for the pathogenesis of otitis due to *Mycoplasma bovis* infection in cattle. Secondly, the mycoplasmaemia developed during acute infection which may involve other organ systems (Gull et al., 2013) including the auditory organs of the sensory system. The fact that viable *Mmm* have been recovered from organs other than those of the pulmonary system provides considerable evidence that this mycoplasma can indeed disseminate from the respiratory tract occasionally (Thiaucourt et al., 2004). Additionally, ear mites have also been associated with potentially pathogenic *Mycoplasma* species of cattle in a CBPP-free country (Santos et al., 2012) as well as with pathogenic *Mycoplasma* species of small ruminants (Francoz et al., 2004; Mercier et al., 2007). The association of ear mites with *Mmm* in the ear canal of cattle in enzootic areas is likely, although in our study ear mite infestation was not investigated. Alternatively, the contamination of the ear canal by *Mmm* in CBPP-infected herds is also possible as previously reported by Corrales et al. (2007) and Arcangiolo et al. (2012) for *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *capri* in goats and *Mycoplasma bovis* infection in calves, respectively.

The *Mmm* isolation rate in this study was low (1.81%). This may be attributed to collection of samples from apparently healthy cattle which is in agreement with Duarte et al. (2003) who reported a significantly lower isolation rate of *Malassezia* species from the external ear canal of apparently healthy cattle compared to those with otitis. It is possible that if apparently sick animals presenting classical signs of CBPP had been sampled, the isolation rate might have been higher. The low isolation rate may also be due to the inferiority of ear canal swabbing compared to ear flushing technique in goats as reported by Ribeiro et al. (1997). On the other hand, the low isolation rate may also be due to the ear canal being an unusual anatomical site for mycoplasma isolation as reported in goats by Cottew and Yeats (1981) and (1982). Perhaps the organism was present in little amounts in ear samples as it is a causative pathogen of a respiratory disease. Also, the low positivity may be due to the study design, unwillingness of herdsmen to allow for sampling and insecurity during the study period. Additionally, constraints such as the heavy presence of antibiotics in the clinical samples, contamination of samples by bacteria and overgrowth by less important but more exuberant mycoplasmas, and the complex requirements for growth may have affected *Mmm* isolation rate (Nicholas et al., 2008; Hassan et al., 2011) in this study because the organism is labile and often present in little quantities (Gedlu, 2004).

In this present study, *Mmm* from ear canal swab samples of apparently healthy cattle in 2013–2016 were detected by PCR in this study. Out of 6 isolates identified as members of the *Mycoplasma mycoides* sub-cluster, 50% (3/6) were confirmed as *Mmm* following the OIE (2014) guideline. These findings are similar to that of Bashiruddin et al. (1994) and Miles et al. (2006).

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Conclusion

In conclusion, the present study is the first in assessing the presence of *Mmm* from the ear canal of cattle in Plateau State, Nigeria. This is of major significance as it implies that the ear canal can be included as a sample collection site for *Mmm* recovery compared to the nasal swab samples which are more prone to contamination by commensal and opportunistic bacteria. The occurrence of *Mmm* in apparently healthy cattle in this study also indicates that it is part of the normal flora of the ear canal.

Acknowledgement

The authors thank the NVRI, Vom for funding this project. The authors also appreciate members of the Mycoplasma Research Group (Sunday Kigbu, Mary Peter Gyang, Emmanuel Damter, Salma Maichibi and Rosemary Goji) and staff of the Applied Biotechnology Division (Olajide Owolodun, Nneka Chima and Anvou Jambo), all of the NVRI for their technical support.

Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Contribution

MAR, HMK and PIA conceived and designed the study. PIA, NJU, IOF, LTI, IAM, PDL and OON collected and analysed samples. MAR, HMK, PIA, PDL, MOO and NDN drafted the manuscript. All authors read and approved the final manuscript for submission.

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