Research Article

Detection of *Mycoplasma bovis* from Cattle Presented for Slaughter in Adamawa and Taraba States, Northeastern Nigeria

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Abstract | *Mycoplasma bovis* is the second most important mycoplasmas causing a variety of diseases in cattle worldwide. This study was aimed to report the PCR identification of *M. bovis* from slaughtered cattle in northeastern Nigeria. Four hundred and eighty (480) samples of lung tissues (180), nasal swab (180), ear swab (60) and pleural fluid (60) were collected from 190 heads of cattle at slaughter in Yola and Jalingo abattoirs in Adamawa and Taraba States, respectively Samples were processed based on standard laboratory protocols. An overall *Mycoplasma bovis* isolation rate of 0.83% (4/480) was obtained. Based on the states studied, 1 (0.35%) and 3 (1.53%) *M. bovis* were isolated from Adamawa and Taraba States, respectively with an insignificant association between *M. bovis* infection and the states sampled (P>0.05). Based on organs/site sampled, 2 (5.40%) isolates of *M. bovis* were from lung tissues and 1 (2.70%) were from both pleural fluid and ear swab samples. All the 4 isolates were confirmed as *M. bovis* by the presence of one band of 734 bp. The study had established first isolation of *M. bovis* from the ear canal of cattle at slaughter in the study area. Therefore, large scale epidemiological studies that will reveal the true prevalence and distribution of *M. bovis* infection in cattle population in Nigeria are recommended.

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Introduction

Mycoplasma bovis (*M. bovis*) is the second most important mycoplasmas of cattle causing respiratory disease, mastitis, arthritis, otitis media, keratoconjunctivitis and a variety of other diseases in cattle worldwide (Brown *et al*., 2015). The organism was first isolated from a cow with mastitis in 1961 in the USA (Caswell and Archanbault, 2007). It lacks cell wall, had a complex nutritional requirement and a low G+C content of 23.8-32.9 mol % (Calcutt *et al*., 2018). It does ferment glucose nor hydrolyses arginine but instead uses organic acids such as lactate and pyruvate as energy source for growth (Maunsell *et al*., 2011; Nicholas *et al*., 2016). *Mycoplasma bovis* has been reported to be transmitted in respiratory secretions through aerosols, nose-to-nose contact, or indirectly via feed, water, housing, or other formite

(Nicholas *et al*., 2002; Parker *et al*., 2018).

Diagnosis of *M. bovis* infection can be performed by serology, isolation and polymerase chain reaction (PCR) on lung tissues (Le Grand *et al*., 2002; Hayman and Hirst, 2003). Diagnosis by culture requires complex media, specialized equipment and technical skills with growth apparent by 48 hours (Nicholas *et al*., 2008). The PCR has been used to detect *M. bovis* from clinical materials with sensitivity higher than that of culture method (Cremonesi *et al*., 2007). Many PCR systems have been routinely described for specific identification of *M. bovis* which target 16S rRNA gene (Bashiruddin *et al*., 2005), *uvr*C gene (Subramaniam *et al*., 1998), *opp*D/F gene (Pinnow *et al*., 2001), and *Vsp* gene (Tenk *et al*., 2006). These had resulted in improved specificity for *M. bovis* detection in samples.

Nigeria is endowed with abundant livestock resources with 19.7 million estimated livestock population and diseases have been reported as a setback in actualization of animal production (Tambuwal and Egwu, 2017). *Mycoplasma bovis* infection is an important emerging disease infecting cattle in Nigeria resulting in high economic losses (Francis, 2017). The disease is poorly understood among livestock owners and animal health handlers, therefore needed to establish the distribution of the disease in the study area. The present study was aimed to detect *M. bovis* from cattle presented for slaughter in Adamawa and Taraba States, northeastern Nigeria.

Materials and Methods

Study area

This study was carried out in two northeastern states of Nigeria, namely; Yola Abattoir in Adamawa State and Jalingo Abattoir in Taraba State (Figure 1). Yola Abattoir based on 3D Global Positioning System (GPS) is located on the Latitude 9°13'33.3"N and Longitude 12°27'10.1"E with an average of 70 - 80 cattle being slaughtered daily and higher during festivals period. Jalingo Abattoir based on 3D Global Positioning System (GPS) is located on the Lattitude 8°54'12.1"N and Longitude 11°21'14.3"E with an average of 40-50 cattle slaughtered daily.

Collection and processing of samples

The samples used for this study were obtained from cattle slaughtered at abattoirs within the study States. The period of collection was from February 2016 to February 2017. A total of four hundred and eighty (480) samples of lung tissues suspected of pneumonia (180), nasal swab (180), ear swab (60) and pleural fluid (60) were collected from 190 heads of cattle at slaughter with cases of suspected pneumonia indicative of *M. bovis* infections (120 from Adamawa State while 70 from Taraba State). Two hundred and eighty-four (284) and one hundred and ninety-six (196) samples (tissues and swabs/pleural fluids) from cattle were collected from the abattoirs in Adamawa and Taraba States, respectively. The samples were transported to the National Veterinary Research Institute Zonal Offices in Yola and Jalingo, respectively where they are processed in 2 ml sterile Nalgene® cryo vials containing 1.5 ml of pleuropneumonia-like organism (PPLO) broth 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.

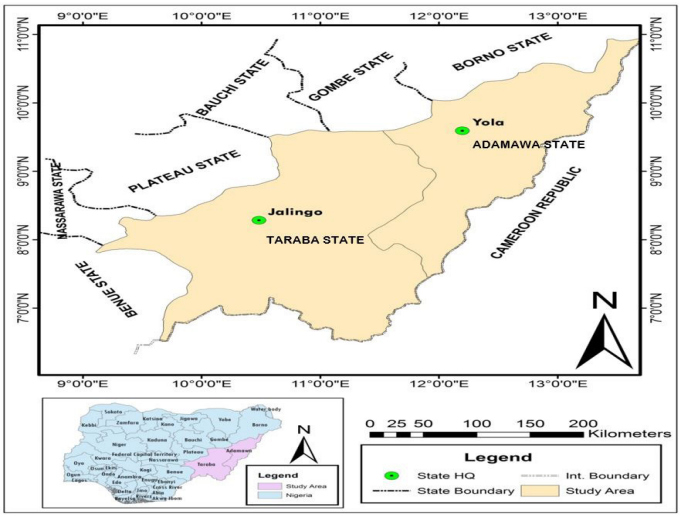

Figure 1: *Map of Adamawa and Taraba States, North-eastern Nigeria (MLS, 2010).*

Microbiological culture of mycoplasmas

The samples were processed according to the method described (Thiaucourt *et al*., 1992). The transport media (PPLO broth) containing various samples were filtered by passing through 0.45µm millipore antibacterial filters which is permeable to organisms smaller than 0.45 µm in sizes such as *Mycoplasma* and viruses. The filtrate was then incubated at 37°C in screw-capped bottles for three (3) days under CO_2 and later cultured onto PPLO agar. These were incubated at 37°C in a 5% CO_2 atmosphere with maximum humidity and examined daily for evidence of growth for 1-2 weeks using a stereomicroscope.

Isolated colonies having the classical 'fried eggs' appearance with dense raised centres (nipples) were triple-cloned by aseptically transferring a small agar block containing an isolated *Mycoplasma* colony into 10 ml PPLO broth and incubated for 72 hours. Both broth and agar media were considered negative if no comet or *Mycoplasma*-like colony was observed after 14 days of incubation. Cloned isolates were stored in triplets at -4°C, -20°C and -80°C. These isolates were later transported under cold chain by FedEx® Red Star Express Courier to OIE Reference Laboratory for Contagious Bovine Pleuropneumonia (CBPP) in Istituto Zooprofilattico Sperimentale dell' Abruzzo e del Molise (IZSAM) "G. Caporale", Teramo, Italy. On receiving the isolates, they were stored at -20°C, the isolates were then removed, verified and then registered under exotic disease unit before being processed.

Detection of Mycoplasma bovis based on 16S rRNA genes

Mycoplasma bovis DNA from the presumptive culture was extracted from 500 µl of broth cultures using a commercially available automated nucleic acid extraction system the Maxwell® 16 Tissue/cell DNA Purification kits (Promega, USA) with Maxwell® 16 instrument (Promega, USA) following manufacturer's instructions. The extracted DNA was resuspended in 500 µl TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 7.6) and was stored at -20°C until analyzed.

The following PCR mix was used for each sample; 25 µl of master mix (Taq Polymerase, dNTPs, $MgCl_2$, Taq Buffer), 18 µl nuclease free water, 1 µl forward primer, 1 µl reverse primer and 5 µl of DNA sample as described by (Bashiruddin *et al*., 2005).

Primers designed for 16S rRNA were used to amplify 734 bp of 16S rRNA gene for *Mycoplasma bovis*. The forward primer was MBOF2: 5'-GAA GCG AAA GTA GCA TAG GAA ATG AT-3' and the reverse primer was MBOR2: 5'-CGT CGT CCC CAC CTT CCT CCC G-3'. The samples were then subjected to amplification reactions consisting of one cycle of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, a final extension step at 72°C for 5 minutes and standby temperature at 4°C. The amplified products were detected by electrophoresis in 1% agarose gel electrophoresis. The resultant bands were stained with ethidium bromide and visualized under ultraviolet light. *Mycoplasma bovis* reference strain PG45 (Wise *et al*., 2011) was used as a positive control, while negative control contained only the master mix.

Data analysis

Data collected were analyzed using simple percentages, while Fisher's Exact test was used to test the level of significance between the variables and the value of $P<0.05$ is considered statistically significant.

Results and Discussion

Thirty-seven (37/480) field isolates of *Mycoplasma* species were identified on culture. Out of which 4 (4/37) yielded positive growth exhibiting fried egg colonies (Figure 2) with a slight presence of film typical of *Mycoplasma bovis* on PPLO agar, giving an overall isolation rate of 0.83% (4/480) (Table 1) while the rest of the isolates do not exhibit typical mycoplasma colonies.

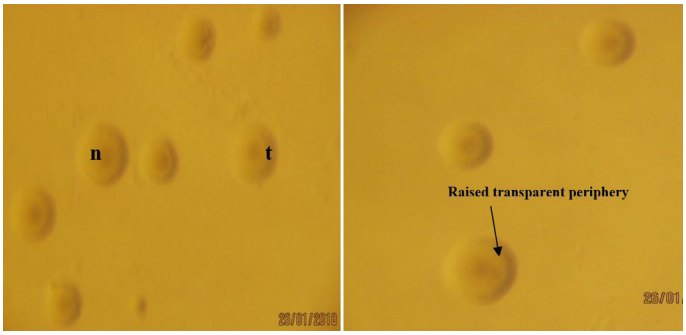

Figure 2: *Colonies of Mycoplasma bovis from lung sample on PPLO agar showing 'fried egg' colonies with nipples 'n' and raised transparent periphery 't' observed under Stereomicroscope (X40).*

Table 1: *Isolation rates of Mycoplasma bovis from slaughtered cattle in Adamawa and Taraba States, northeastern Nigeria.*

Source of examined isolates	No. of samples	No. of M. bovis identified (%)	P-value
States			
Adamawa	284	1 (0.35)	
Taraba	196	3 (1.53)	0.3095
Organs			
Lungs	180	2 (1.11)	
Pleural fluid	180	1 (0.56)	
Nasal swab	60	0 (0.00)	
Ear swab	60	1 (1.67)	0.8409
Total	480	4 (0.83)	

Based on the two States sampled, 1 (0.35%) and

3 (1.53%) isolates of *M. bovis* were isolated from Adamawa and Taraba States respectively with no statistically significant difference observed between *M. bovis* infection and the states sampled (P>0.05). Based on organs/site sampled, 2 (5.40%) isolates of *M. bovis* were isolated from lung tissues and 1 (2.70%) was isolated from both pleural fluid and ear swab samples while no *M. bovis* isolate was identified from nasal swab with an insignificant statistical difference (P>0.05). *Mycoplasma bovis* was recovered from lung tissues, pleural fluid and ear swab samples collected (Table 1).

The four (4) isolates were confirmed by PCR based on 16S rRNA gene as *Mycoplasma bovis* by the presence of one band of 734 bp at the same distance with positive control that was used (Figure 3).

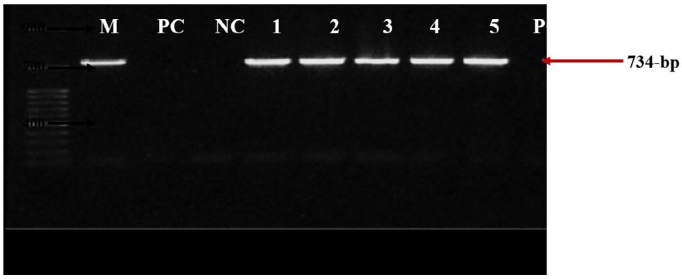

Figure 3: *PCR amplification of Mycoplasma bovis 16S rRNA gene on 1% agarose gel with primers MBOF2 and MBOR2. M: molecular marker of 50-bp; PC: positive control reference strain PG45 (734-bp); NC: negative control; Lanes 1-5: represents samples TL41, TL43, TL46, TES57 and AP106.*

Mycoplasma species are pathogenic *Mollicutes* affecting animals, humans and plants and therefore of great concern in human and veterinary medicine (Saad and Abdel-Hameed, 2012). The isolation and identification rate of 0.83 % (4/480) of *M. bovis* reported in this study is lower than 2.0 % isolation by (Francis *et al.*, 2014) from pneumonic lungs of cattle from Adamawa State and 8.9 % isolation (Tambuwal and Egwu, 2017) from pneumonic lungs of cattle from two northwestern States of Nigeria. The difference in the isolation rates may be attributed to the delayed analysis of the samples after collection. The isolation of *M. bovis* from pneumonic lungs of slaughtered cattle shows that *M. bovis* and its interrelated infection may be occurring in the study area. This finding is of clinical significance because these few infected cattle could likely be a threat and basis of infection to several vulnerable and in-contact herds and farms in the study area and hence leading to increase in prevalence. Similar observations were made (Egwu *et al.*, 1996; Ajuwape *et al.*, 2003; Francis *et al.*, 2014; Tambuwal and Egwu, 2017).

Mycoplasma bovis infection was identified to be higher in Taraba State than in Adamawa State even though there was an insignificant statistical difference (P>0.05). This signifies a higher risk of susceptibility to infection with *M. bovis* for cattle in Taraba State than their counterpart in Adamawa State, even though there was a strong link in the movement of cattle between the two states in search of forage and favorable market. Similar findings have been reported by previous authors (Tambuwal *et al.*, 2011; Francis *et al.*, 2014).

Based on organs/site sampled, *M. bovis* was identified higher in lung tissues than pleural fluid and ear swab. This might be attributed to the fact that, the predilection site for most *Mycoplasma* species is the respiratory system (Arcangioli *et al.*, 2008), where these organisms multiply and cause pathological lesions (Nicholas *et al.*, 2008). Dissemination to other organs and parts of the body occurs as the infection progresses to a chronic stage leading to the recovery of these pathogens in the pleural sac, nasal and ear canals (Santos *et al.*, 2009).

Mycoplasma bovis was isolated and identified from the ear canal of cattle in Taraba State with a 1.67% isolation rate. This is of scientific significance as the organism has been previously reported to cause otitis media in cattle (Alberti *et al.*, 2006). To the best of our knowledge, this is the first report of isolation of *M. bovis* from the bovine ear canal in Nigeria. The presence of the *M. bovis* in these sites is not surprising in view of the fact that they are widely distributed in nature. Considering the ability of other ruminant mycoplasmas to colonize the ear canal, ear involvement by *M. bovis* may not be doubtful. The assumptions on the occurrence of *M. bovis* in the ear canal include the anatomical connection between the pharynx and middle ear via Eustachian tube for the pathogenesis of otitis media due to *M. bovis* infection in cattle (Maeda *et al.*, 2003; Lamm *et al.*, 2004; Arcangioli *et al.*, 2012). Also the mycoplasmaemia developed for the period of acute infection may involve dissemination of mycoplasmas from primary site of infection (the respiratory system) to other organs and systems including the auditory organs where the organisms were isolated (Gull *et al.*, 2013). The report was consistent with previous report (Santos *et al.*, 2009) who also for the first time reported *M. bovis*

and a crowd of other mycoplasmas from the ear canal of cattle in Brazil.

Molecular identification of isolates from broth culture, identified 16S rRNA gene of *M. bovis* isolates TL43, TL46, TES57 and AP106, and was consistent with previous reports (Johansson *et al*., 1996; Bashiruddin *et al*., 2001, 2005).

Conclusion

The present study has demonstrated *M. bovis* from lung tissues, pleural fluid and ear canal of cattle and thus, the first report of isolation of *M. bovis* from an ear canal of cattle at slaughter in the study area. We therefore, recommend large scale epidemiological studies that will reveal the prevalence and distribution of *M. bovis* infection in cattle populations in Nigeria.

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Authors' Contribution

CNK, JA, LA, MAR, GOE and MIF conceived and designed the study. PIA and MIF collected and analyzed samples. FS, MS and MIF performed molecular identification. MIF, PIA, MAR and FS drafted the manuscript. All authors read and approved the final manuscript for submission.

Conflict of interest

The authors have declared no conflict of interest.

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