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Research Paper

Occurrence of *Mycoplasma Mycoides* Subspecies *Mycoides* Small Colony (*Mmm SC*), *Mycoplasma Bovis* (*M. bovis*) from the Respiratory Tracts of Cattle Presented for Slaughter in North-western Nigeria

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This study was conducted to determine the presence of *Mycoplasma mycoides subspecies mycoides* small colony and *Mycoplasma bovis* along with associated bacteria in cattle presented for slaughter in Sokoto and Kebbi States, North-western Nigeria. A total of 310 lungs, (155) each from apparently normal and pneumonic lungs were collected from slaughtered cattle and examined for the presence of *Mmm SC* and *M. bovis* using cultural characteristics, biochemical tests and molecular (PCR) test for the confirmations of mycoplasma isolates only. *Mmm SC* were detected in 3.8% and 6.9% of normal and pneumonic lungs respectively. Similarly, 6.2% and 8.9% were detected for *M.bovis* in normal and disease lungs. Furthermore, nested PCR detected 78 (51.3%) and 56 (36.8%) of *Mmm SC* and *M. bovis*, respectively. The study established the occurrence of both *Mmm SC* and *M. bovis* for the first time in cattle presented for slaughter in Sokoto and Kebbi States. The study confirmed the presence of CBPP and *M. bovis* related infections in the study area.

Key words: Occurrence, mycoplasma, cattle, abattoir, Nigeria.

INTRODUCTION

Nigeria is one of the leading countries in cattle production in Sub-Saharan Africa with a population of approximately 19.5 million (NASS, 2011). The production of these ruminant livestock constitutes an important component of Nigeria's agricultural economy (Tolulope and Chinonso, 2013). According to Benard et al. (2010), Nigerian cattle industry generates USD 6.8 billion of a potential USD 20 billion annually. However, majority of these animals are managed under extensive system with a few subjected to intensive and semi-intensive systems of husbandry (Obadiah and Shekaro; 2012, Ajah, 2012, Olorunnisomo, 2013). Some of these traditional systems of management are counterproductive as it exposes the animals to stressful condition and threat of diseases (Ikhatua, 2011). For decades, livestock diseases have been identified as major constraints to improved livestock productivity in Nigeria (Griffen, 1978; Lamorde, 1996; Williams et al., 2000). Of particular concern are those diseases affecting cattle that cause severe infections particularly in endemic areas (Sayin et al., 2016). In bovine, the two most important Mycoplasmal disease agents are *Mmm SC* and *M. bovis* (Nicolet, 1996; Maksimovic and Rifatbegovic, 2012; Admassu et al., 2015), and can affect cattle singly or in synergy with other microbial agents (Alberto et al., 2006). Whereas the former is known to cause Contagious Bovine Pleuropneumonia (CBPP) in calves and adult

cattle (Egwu et al., 2012; Kairu-Wanyoike et al., 2014; Musa et al., 2016), the latter is known to cause clinical disease syndrome such as pneumonia, mastitis, polyarthritis, reproductive disorders and occasionally other diseases of low incidence in the affected cattle (Levisohn et al., 2004, Arcangioli et al., 2011; Aebi et al., 2015). The diseases caused by the duo agents are serious economic and veterinary health problems that threaten food and social security particularly in Africa south of the Sahara (Kairu-Wanyoike et al, 2014, Musa et al., 2016). In Nigeria, available veterinary records have confirmed the endemicity of CBPP and is wide spread amongst cattle population in pastoral herds, institutional ranches, dairy farms, government livestock breeding centres and abattoirs (PACE, 2004, Babalobi, 2007, Danbirni et al., 2010, Tambuwal et al., 2011; Egwu et al., 2012) with varying prevalence that ranges between 0.15% and 47%). This epidemiological spread illustrates the need for control measures to be in place for both infected and naive zones of the country.

Comparatively, the prevalence and pattern of spread of *M. bovis* infections in the country may be different due to paucity of epidemiological data except isolated investigative reports (Egwu et al., 2012). Currently, information on the actual prevalence and impact of *MmmvSC* and *M. bovis* in the country is uncertain and there are no published reports of these organisms being detected in indigenous breeds of cattle in the study States. Documented information on these important mycoplasma pathogens could help to better the understanding on how to institute control and preventive measures against diseases associated with them. The present study was therefore designed to detect the occurrence of these pathogens (mycoplasma) in cattle presented for slaughter in the study area.

MATERIALS AND METHODS

STUDY AREA

Sokoto and Kebbi States are located in the north-western part of Nigeria. Geographically, the two States share borders and are located between latitudes 10° and 14° N, and longitudes 3° and 7° E (Abdullahi, 1985). The States share international boundary with republic of Niger in the North while Kebbi State borders with Benin Republic in the west, the two States are bounded by Niger and Zamfara States of Nigeria in the south and east, respectively (Figure 1). The two States (Sokoto and Kebbi) are rich in terms of livestock resources accounting for 6,110,000 (37.48%) of the standing cattle population in the country (Ikhatua, 2011). The climate in the area is semi-arid in nature coupled with severe rain scarcity from October to May and becomes available only in July to September with an annual average rainfall being less than 30 inches (RIM, 1992). The mean monthly temperature ranges between 13°C in December through February, and 40– 42°C in April and May. The relative humidity in the area varies from 10% in February to 90% in August. The main occupation of the people is arable farming and livestock rearing with cattle, sheep and goats predominating. Livestock production is under-taken by both settled, semi-settled farmers and pastoralists.

Sample collection

The tissues (lungs) used for this study were obtained from cattle slaughtered at central abattoirs within the study States. The period of collection was from December, 2013 to June 2014. The slaughter houses were visited twice a week (Mondays to Fridays) for sample collection. Lung samples from areas of congestion, consolidation and oedema lesions (pneumonic) and apparently normal (non-pneumonic) were collected from each animal for bacteriological examination. A total of 310 samples were collected in parallel comprising 155 each from pneumonic (diseased) and apparently normal lungs out of approximately 23,000 cattle slaughtered and examined at post-mortem. These lung tissues were used for the determination of bacterial and mycoplasma flora in unaffected and affected mycoplasma lesions. The tissues were properly labelled, packaged and transported to Mycoplasma Laboratory, Veterinary Medical Research Institute, Budapest Hungary. The cattle examined in this study were local breeds normally presented for slaughter comprised Gudali, Rahaji, White-Fulani and their crosses. They were aged 1- 14 years using standard procedure (Lasisi et al., 2002), and were managed semi-intensively, fed with dry legume hay/pasture and supplemented with local concentrate (Wheat offal).

Laboratory culture for suspect mycoplasma samples

Suspected tissue (lung) samples were cultured and sub-cultured on modified Eaton's medium according to standard procedures in the Central Veterinary Laboratory's manual on Mycoplasmology (Boughton and Thorn, 1993). Inoculated broth and agar plates were incubated at 37°C in 5% carbon dioxide for up to three weeks. The inoculated broth medium which showed a colour change from pink to yellow as denoted by a colour change by half of a PH change of 0.5 units were sub-cultured into a fresh broth medium and onto agar plates. Following further incubation, representative colonies were then sub-cultured by agar push technique. Representative mycoplasma colonies in single or mixed cultures were identified by Growth Inhibition test (GI) and Indirect Immunoflourescent test (IFA) using type specific antisera. Concisely, the GI test involved adding of an over-night broth culture of the Mycoplasma culture onto an agar

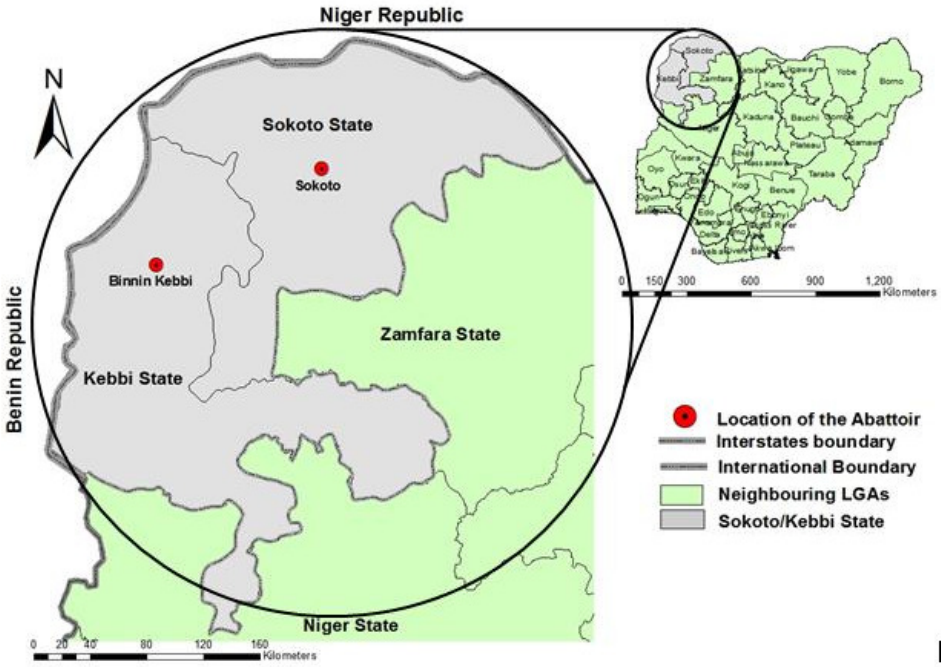

Figure 1. Map of Sokoto and Kebbi States showing boundaries (national and international) and the locations of the abattoirs used for the study.

plate, and allowing the drop to run down the plate as a lane when slightly tilted. Then specific antisera impregnated to a disc were placed at the centre of the lane. The plates were incubated at 37°C and checked for inhibition of growth for the specific mycoplasmas. Growth inhibitory zones of ≥ 2 mm were considered positive. The IFA technique entailed essentially of washing cut agar bearing mycoplasma colonies following addition of specific antisera and fluorescent labelled antiglobulin (conjugate). These washed colonies reacted with specific antisera and conjugate were visualized under the UV light for greenish yellow fluorescent as described by Rosendel and Black (1972).

The typical fried egg appearance colonies of mycoplasmas were observed on solid media. For bacterial culture and identification, samples from pneumonic and normal lungs were directly placed into peptone water and incubated over night at 37°C as pre enrichment. A loopful from each of the peptone water was then cultured onto MacConkey agar (MCA) Oxoid UK and 5% sheep blood agar (SBA) plates. All the cultured plates were incubated aerobically and anaerobically at 37°C for 24 – 48 h. Suspect colonies were carefully picked and sub cultured on blood agar and nutrient agar and incubated for 24 h at 37°C to obtained pure bacterial cultures. A stock culture from each isolate was then stored at 4°C and later identified based on morphological appearance and Grams stain. Confirmation of isolates was carried out according to standard biochemical procedures (Markey, 2013).

DNA extraction and PCR of mycoplasma isolates

From the previously frozen and homogenised lung tissues, 10 percent suspensions of lung tissues in phosphate buffered saline were thawed at 4°C and 500 µl aliquots of suspensions were centrifuged for 10 min at 14000 g. The pellets were washed once with 500 µl of 10 Mm Tris-Hcl (pH 8.0), 10 Nm sodium chloride, 10 Mm EDTA buffer. The DNA was extracted by the method described by Bashiruddin et al., 1994a. The precipitated DNA from each sample was resuspended in 10µl of deionised PCR grade water and 1µl was used for each amplification reaction. The amplification was carried out in the Gene Amp 9600 PCR system (Pekin-Elmer) with the *M. mycoides* cluster-specific primers (MC323/MC358), and M. mycoides-specific primers provided in the pleuroTrap kit (Silenus), and *M. bovis*-specific primers (MboF2/R2). These primers pairs amplified about 1.5kb, 0.5kb and 0.7kb regions of the genome respectively (Bashiruddin et al., 1994b; Johansson et al., 1996). As controls, either DNA prepared from Mmm SC Afade strain or Italian field strain 57/13, the positive control provided in the pleura TRAP kit, or *M. bovis* strain Donetta DNA were used, as appropriate. The amplified mycoides cluster-specific and *M. bovis*-specific products were analyzed by gel electrophoresis on 1 percent agarose containing ethidium bromide. Amplified products of *M. mycoides*-specific primers were detected with the pleuroTRAP kit, using the calorimeter micro plate system (Silenus) according to the manufacturer's instructions.

Colour production indicated the presence of *M. mycoides* group in the samples. The fragments were identified as Mmm SC by digesting the PCR products with restriction enzyme Ans1 and separating them in 3 percent agarose gel as two bands of 380 bp and 180bp (Bashiruddin et al., 1999b).

Statistics

The data was analyzed using simple percentages, while Chi-square was used to test the level of significance at a probability level of P = 0.05).

RESULTS

In general, bacteria of different genera /species were isolated with varying frequencies among all the lung tissues sampled. The occurrence of common Mycoplasmal and bacterial isolates is presented in (Table 1). A total of 970 bacterial and 152 mycoplasma isolates were isolated from all the animals investigated. The breakdown of the figures showed that 730 bacteria species were isolated from pneumonic lung against 392 from apparently normal lungs (P< 0.05). Similarly, 110 mycoplasma species were obtained from pneumonic lungs compared to 42 from apparently normal lungs (P < 0.05). *Mmm SC* and *M. bovis* each accounted for 6.2% and 8.9% of the total isolates from pneumonic lungs against 3.8% and 6.9% in apparently normal lungs. There were different bacterial species isolated and it is notable that most bacterial pathogens causing pneumonia in the affected cattle were similar to those detected in healthy animals and the distribution rates is shown in (Table 1). Overall, the results indicated that more mycoplasma and bacteria were isolated from pneumonic lungs compared to apparently normal lungs with significant difference (P < 0.05) (Figure 2).

DISCUSSION

The role of Mycoplasmas in the aetiology of infectious diseases in most species of animals including wildlife has long been established (Jones, 1983, FAO, 2002, Nicholas, 2004, Chalker et al*.,* 2004; Sumithra et al., 2013). However, diagnosis of Mycoplasmal infections is not straight forward and infections are frequently under diagnosed and misinterpreted. Research reports (Egwu et al., 1996; OIE, 2014) have linked constraints to accurate diagnosis in the affected animal to the manifestations of multiple clinical and pathological signs in the affected animal occasioned by cross-reactivity caused by related mycoplasmas. This sometimes creates confusion and doubt in concluding diagnosis in the herds leading to persistent disease problems typical of mycoplasma infections (Nicholas, 2004).

Findings from this study have shown that variety of species of mycoplasmas and bacteria colonise the lower respiratory tracts of cattle. Mycoplasmas were isolated from both normal and pneumonic lungs. The incidence of 3.8% and 6.9% in respect of *Mmm SC* and *M. bovis* from apparently normal lungs and, 6.2% and 8.9% for Mmm SC and *M. bovis* in pneumonic lungs recorded in this study is of clinical importance because these carrier animals could be a threat to healthy in-contact cattle. The isolation rate of mycoplasmas recorded in this study could have been high than this taking into consideration that most farmers in the area treated cattle against respiratory diseases (Tambuwal et al., 2011) which might suppress the development of pulmonary lesions. According to Stipkovits et al. (2001), the increasing use of antibiotics by farmers is an attempt to reduce the suffering of the affected cattle with respiratory infection, promote quick recovery as well as maintenance of carriers in endemic areas.

Likewise, the isolation of *Mmm SC* and *M. bovis* pathogens from cattle originating from Sokoto and Kebbi States evidently shows that CBPP and *M. bovis* related infections are very active in these areas. Perhaps, the irregular vaccination programme occasioned by low vaccination coverage might have been responsible for the sustained spread of these infections in the area notably CBPP. The detection of *Mannheimia haemolytica*, *Pasteurella multicida* and *Histophilus somni* in these animals under traditional system of husbandry should be a source of concern. The three (Bacteria) frequently associated with bovine respiratory disease complex (BRDC) are opportunistic pathogens residing in the respiratory tracts of healthy cattle and probably becoming pathogenic when stress and secondary infection impairs immune function (Griffin et al., 2010). Caswell, (2014) has shown that Pneumonia caused by opportunistic pathogens is common and important in all domestic animal species and humans.

In the present study, most bacteria isolated from normal lungs were similar to those isolated from pneumonic lungs with significant difference (P < 0.05). Immunocompromisation due to respiratory defence's failure could have been responsible for high number of bacterial population recorded in the lungs of pneumonic cattle (Czuprynski, 2009). In sharp contrast, the relatively small number of bacteria population in apparently normal lungs suggests that the natural barriers of these cattle are able to keep the normal flora in check (Ajuwape and Aregbesola, 2002). The reported 5.2% and 10.1% detection rate of *Pasteurella multicida* from the respiratory tracts of apparently normal and disease cattle examined was lower than the one reported by Ojo (1976) in goats. In all the bacteria isolated, Staphylococcus species (*S. aureus* and *S. albus*) occurred in high numbers in both apparently normal and pneumonic lungs. As has been pointed out (Thomson, 2000) *Mammheimia*

Table 1. Mycoplasma and bacteria isolated from apparently normal and pneumonic lungs of traded cattle presented for slaughter at abattoirs in the study states.

Mycoplasma/Bacterial species	Isolates from apparently normal lungs (n=155)	Isolates from pneumonic lungs (n=155)
Mycoplasma mycoides subspecies mycoides SC	3.8%)[a]	45 (6.2%)[b]
Mycoplasma bovis	27 (6.9%)[a]	65 (8.9%)b
Escherichia coli	7 (1.8%)[a]	24 (3.3%)[b]
Staphylococcus aureus	64 (16.3%)[a]	72 (9.9%)[a]
Staphylococcus albus	44 (11.2%)a	60 (8.2%)[b]
Mannheimia haemolytica	23 (5.9%)	67 (9.2%)
Pasteurella multicida	15 (3.8%)[a]	48 (6.6%)[b]
Histophilus somni	19 (4.8%)	71 (9.7%)
Corynebacterium pyogenes	21 (5.4%)	38 (5.2%)
Streptococcus spp	60 (15.3%)[a]	52 (7.1%)[a]
Aerobacter aerogenosa	0	32 (4.4%)
Bordetella spp	23 (5.9%)	28 (3.8%)
Klebsiella pneumonia	32 (8.2%)[a]	12 (1.6%)[b]
Branhamella spp	32 (8.2%)	48 (6.6%)
Candida albicans	0	32 (4.4%)
Micrococcus spp	10 (2.5%)	36 (4.9%)
Total	392[a]	730[b]

Figures in brackets are percentage prevalence of mycoplasma and bacterial species.
Values denoted by different superscripts for a given parameter are significantly different (P = 0.005; P< 0.05)

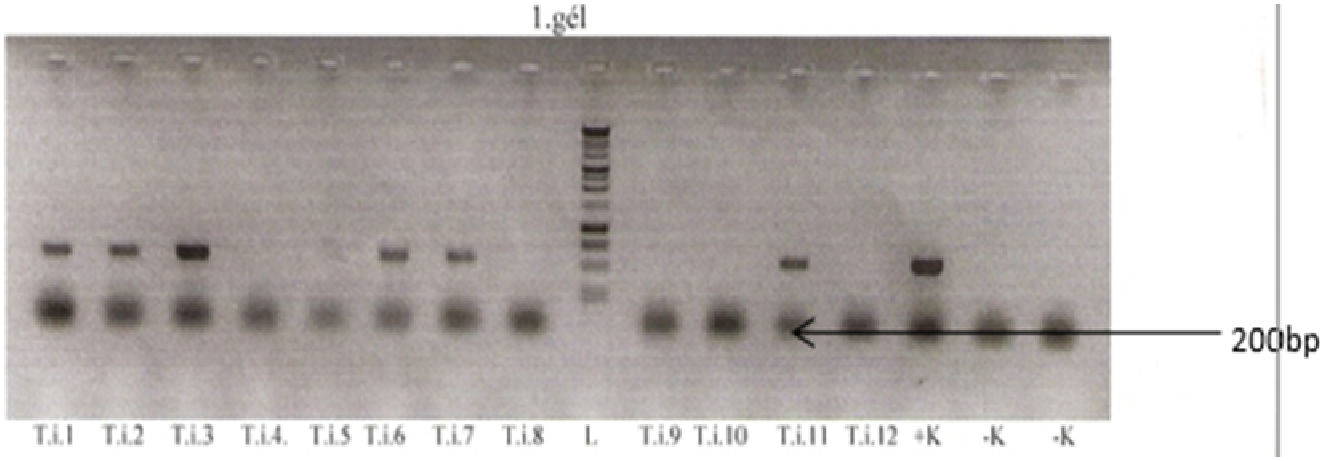

Figure 2. Gel electrophoresis in 1 percent agarose of PCR productswith primer pair MboF2/R2 of some *M. bovis* isolates from cattle. Lane L Molecular size markers fanging from 2000 to 50 bp , Lanes Ti9,Ti10, Ti11, Ti12 were positive isolates.Lane +K,Positive control, Lane –K Negative control.

haemolytica, *Pasteurella multicida* and *Histophilus somni* are common pathogens of bovine nasal flora, yet these bacteria can cause devastating respiratory disease (Shipping fever) in cattle.

Pneumonia associated with these bacteria often occurs when the animal normal defence is compromised (Irsik, 2007). The detection of enterobacteriaceace group (*E. coli* and *Klebsiella pneumoniae*) in the respiratory tracts of apparently normal and diseased cattle is interesting. The isolation of *E. coli* from apparently normal cattle without apparent clinical enteritis suggests that they are also natural habitat of the respiratory tract of cattle, but can cause disease depending on the serotype and the host defence (Quin et al., 2016). The only opportunistic fungus isolated was *Candida albicans* in pneumonic lungs only. Candida spp are normal inhabitant of alimentary, upper respiratory and genital mucosae of animals and are mostly responsible for their pathology (Glenn Songer and Post, 2005). The overall predominance of a variety of bacterial and fungal organisms could seriously influence the pathogenesis and pathology of CBPP and *M. bovis* associated respiratory infections. Sokoto Gudali was the predominant breed encountered during the study accounting for 56.8% of the total cattle bled. Gudali breed is an indigenous breed of cattle found predominantly in the North western zone of Nigeria. The breed represents 32% of the national herd with favoured characteristics of

high carcass and milk yields (Olorunnisomo, 2013; Okeh and Uguru, 2014) as well as pulling of plough for tillage (Kubkomawa, 2017). Ironically, the presence of *Mmm SC* and *M. bovis* pathogens singly or with other concurrent infections in these cattle may have some synergistic roles in precipitating CBPP (Stipkovits et al., 2001).The authors furthermore, reported the existence of resemblances in the pathogenesis and pathology of *Mmm SC* and *M. bovis* infections.

Conclusion

Samples collected and analyzed in this study showed the presence of *Mmm SC* and *M. bovis* in both healthy and diseased animals. The chances of spread of these agents to naïve cattle are high affecting both small-scale and commercial farmers with the resultant economic damage. As a control and preventive measures, there is urgent need to quarantine of all new animals before introduction into the herds or farms. New stock should be sourced from CBPP and *M. bovis* free areas. Desirable also is the need to Increase post-mortem surveillance at abattoirs/slabs to monitor the status of the diseases. Large scale sero-monitoring exercise of cattle in pastoral herds and institutional farms should be undertaken by the relevant authorities to assess the level of infection of the diseases in the country. The concern ministry should educate cattle owners (Herders) on the importance of animal movement restriction, prompt report of cases and outbreaks to nearest authority as well as regular presentation of their stock for CBPP mass vaccination.

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