

Full Length Research Paper

Novel Alleles 8 and 9 Strains of *Mycoplasma mycoides* subsp. *mycoides* circulating in South East Nigeria and Comparison with Vaccine Reference Strain T1/44

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Control of contagious bovine pleuropneumonia (CBPP) is difficult in Nigeria due to lack of sufficient data. *Mycoplasma mycoides* subsp. *mycoides* is the causative organism of CBPP. This disease is endemic in Nigeria with serious economic impact. This study was undertaken to characterize *M. mycoides* subsp. *mycoides* (*Mmm*) in cattle in south-east Nigeria using single-locus sequence analysis based on polymorphism analysis of non-coding genes and also comparison of the obtained sequences with the vaccine reference strain (T1/44) that is presently used in Nigeria. Twenty one Polymerase chain reaction (PCR) confirmed *Mmm* isolates from Anambra and Enugu states of Nigeria were used for this study. The amplicons were amplified using one locus of the PG1 reference strain (LocPG1-0001) and sequenced using the corresponding primers. The sequences were edited using BioEdit™ software and a consensus sequence obtained. Thereafter, the consensus sequence was aligned using ClustalW (BioEdit™). The consensus sequences obtained were compared with the PG1 reference strain and allele numbers were assigned based on any nucleotide changes. Three allelic numbers were obtained; Allele 1, 8 and 9. Allele number 8 and 9 are new findings as they were not previously reported in Nigeria. Points of polymorphism were observed between the vaccine strain and the field isolates. The 21 sequences were deposited in the Genbank with the following accession numbers; MW487814-MW487834.

Key words: Alleles, multi-locus sequence analysis, *Mycoplasma mycoides* subsp. *mycoides*, Nigeria, polymorphism.

INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the causative organism of contagious bovine

pleuropneumonia (CBPP) (Thiaucourt et al., 2006). It is a highly contagious and fatal disease of cattle, causing

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serious economic losses in Nigeria and some African countries (Egwu et al., 1996; Fadiga et al., 2013). CBPP is a trans-boundary disease (TAD) and is one of the bacterial diseases in the OIE list A diseases (OIE, 2000). The disease negatively affects cattle production in Nigeria and Africa (Egwu et al., 1996). There is an upsurge in the incidence of CBPP in Nigeria especially in the south east region (Anyika et al., 2021). So many pastoralists have adopted 'the live with the disease' perspective in Nigeria (Chima et al., 2001). This has resulted in the heavy usage of antibiotics for the treatment of the disease and reporting it (Chima et al., 2001). Several authors have documented the economic impact of CBPP in Nigeria (Aliyu et al., 2003; Fadiga et al., 2013; Tambi et al., 2006). Successful isolation and identification of the causative organism, is the first major step in designing an effective control program of the disease. *M. mycoides* subsp. *mycoides* is a member of the *M. mycoides* cluster, made up of six mycoplasma subspecies, closely related and at a point thought to be homogeneous (Cottew et al., 1987). However, with the advent of different molecular technologies, the identification and differentiation of the different *Mycoplasma* strains is possible (Miles et al., 2006; Lorenzon et al., 2003). Miles et al. (2006) developed an efficient polymerase chain reaction protocol that could differentiate the European strains of *M. mycoides mycoides* from the African strains, by identification of an 8.8 kb deletion in the genome of most *M. mycoides* Subsp *mycoides* strains of European origin. Lorenzon et al. (2003), identified 15 different allelic profiles of *Mmm* from 48 different strains using a technique that involves sequencing multiple loci, termed Multi Locus Sequence Analysis (MLSA). Similarly, Yaya et al., (2008) also identified three main groups and 31 different allelic profiles of *Mmm* using the same method. Multi-locus Sequence analysis is a technique that is based on sequencing multiple loci. It is a very robust approach (Yaya et al., 2008).

In Nigeria, Nwankpa (2008) identified three different allelic profiles of *Mmm* circulating in Northern Nigeria. However, the study was not extended to other parts of the country. This current study is aimed at characterizing *Mmm* isolates from the south-eastern part of Nigeria and comparing the sequences obtained with the vaccine strain (T1/44). This is very important in designing an effective control programme of CBPP in Nigeria. Several reports have indicated the in efficiency of the current vaccine (T1/44) in protecting cattle against CBPP in the field (Thiaucourt et al., 2006; Yaya et al., 1999).

MATERIALS AND METHODS

Study area

This study was conducted in three selected South Eastern states of Nigeria (Anambra, Enugu and Imo states). It is one of the six geopolitical zones in the country. The region consists of Anambra,

Enugu, Imo, Ebonyi and Abia states (Figure 1). 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. South-east Nigeria has an estimated cattle population of 4.5 million from the total of 16.3 million estimated cattle population in Nigeria (Ikhatua, 2011).

Confirmation of *Mmm* isolates using polymerase chain reaction (PCR)

Twenty one *Mycoplasma* isolates from the two South-Eastern states of Nigeria (11 from Enugu state, 10 from Anambra state), positive on Pleuropneumonia like organism (PPLO) agar were confirmed using conventional PCR according to protocols by Miles et al. (2006). DNA was extracted from a 5 ml *Mmm* broth culture using QIAamp® DNA Mini kits according to manufacturer's instructions. Lyophilized T1/44 vaccine (NVRI, Vom Nigeria) was used as the positive control for this reaction. All the PCR reactions were carried out in a total volume of 25 µl, that consisted of dH₂O, 5x FIREPol® master mix (12 mM MgCl, 1 mM dNTP mix, FIREPol® DNA polymerase and 1 µl IS1296F: Primer (5' to 3'): CTA AAG AGC TTG GAG TTC AGT G and 1 µl R (all) (sequence 5' to 3'): CCA GCT CAACCA GCT CCA G (Miles et al., 2006).

DNA amplification was performed using GeneAmp® PCR system 2720 (Perkin Elmer, Courtaboeuf, France) with an initial denaturation step at 95°C for 5 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min and 20 s. The final extension stage was maintained at 72°C for 5 min. The PCR product was then ran on 2% agarose gel impregnated with ethidium bromide (0.5 µg/ml) at 130 volts for 30 min. Finally, the DNA migration was viewed under ultra-violet light and photographs 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 mycoides*.

Molecular characterization of *Mmm* isolates

All the twenty one PCR confirmed isolates were characterized using Single-Locus Sequence Analysis according to protocol of Yaya et al. (2008). It was carried out using the locus: Loc- PG1-0001(non-coding region). This locus was selected due to its ability to effectively differentiate *M. mycoides mycoides* strains from West Africa (Yaya et al., 2008). In brief, PCR reaction was performed using GeneAmp® PCR system 2720 (Perkin Elmer, Courtaboeuf, France) in a final volume of 50 µl reaction mix. Samples were amplified with the following primers: Loc-PG1-0001-F: 5'AACAAAAGAGATCTTAAATCACACTTTA 3' and Loc-PG1-0001-R: 5' CCTCTTGTTTAACTTCTAGATCAGAAT 3 (Yaya et al., 2008). Thermal cycling involved an initial step of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 90 s. The final extension step was maintained at 72°C for 7 min. The PCR amplification products was then analyzed by electrophoresis through 1.5% agarose gel (QA-Agarose, MP Biomedicas, IllKirch, France) at 100V and viewed after staining with Ethidium bromide on a ultra-violet trans-illuminator. Subsequently, samples with the expected band size of 538 base pairs (Figure 2) were sent to Macrogen™ Europe B.V, The Netherlands, for sequencing.

Sequence analysis of amplicons

The sequences obtained from Macrogen™ Europe B.V with the

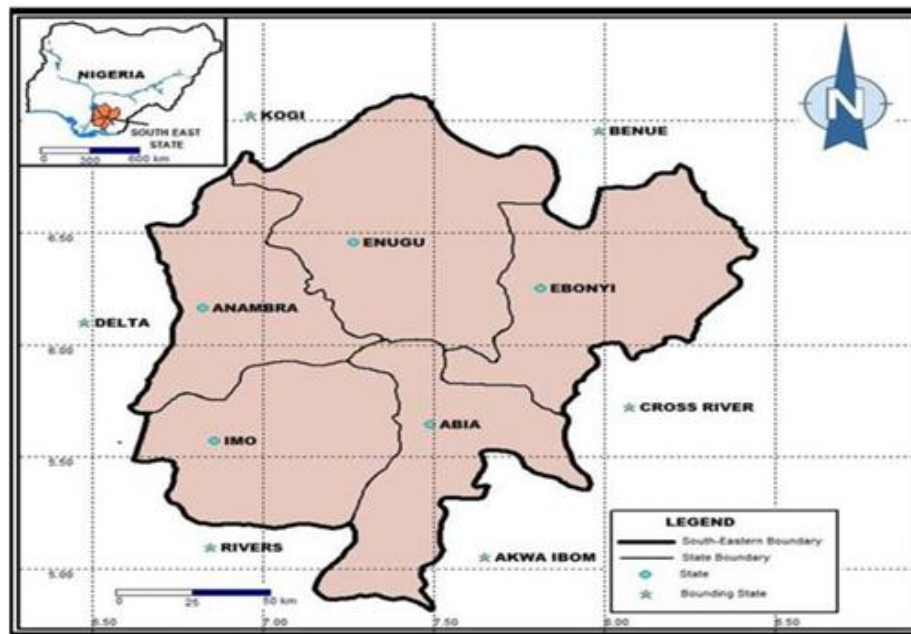


Figure 1. Map of Nigeria showing the South-eastern states of Nigeria.

forward and reverse primers of each sample was assembled using BioEdit™ software. Thereafter, the extremes of each sequence or those showing aberrant features were cut off by carefully examining the chromatographs, after which a consensus sequence was obtained. The consensus sequences obtained from the different isolates for the selected locus (Loc-PG1-0001) was aligned using ClustalW (BioEdit™). Subsequently, the sequences obtained were compared with the PGI reference genome in the Genbank and those obtained from the 51 strains of *Mmm* by Yaya et al. (2008). If the feature of a strain corresponds to one of the strains in the work of Yaya et al. (2008), its allele number was given to the strain. However, if there are differences, a new allele number was given. The strains were characterized using allele numbers. Furthermore, the consensus sequences obtained for each sample was also compared with the vaccine reference strain (T1/44). Points of polymorphism between the field isolates and vaccine strain were documented.

RESULTS

Multi-locus sequence analysis on Loc-PG1-0001

Twenty one of the PCR confirmed *M. mycoides* subsp *mycoides* isolates were of the expected band size of the selected locus, Loc-PG1-0001 as indicated by the production of a band size equivalent to 538 bp (Figure 2).

Alleles identified in the non-coding sequences Loc-PG1-0001

Three allele numbers were identified on the locus Loc-PG-0001 (allele 1, 8 and 9) (Figures 3 and 4). There was no point mutation at position 1523 on the PG1 reference

genome. All the isolates had A (Figure 3) (Allele 1). However, there were point mutations on two positions: position 1525 and 1751 on the PG1 reference genome. Isolate A8 had A at position 1525 of the PG1 genome while the others had T (Figure 3) (Allele number 8). Similarly, isolate A3 had A at position 1751 while the other isolates had T (Figure 4) (Allele number 9). Allele 1 was found in both Anambra and Enugu state while Allele 8 and 9 were only found in Anambra state. Alleles 8 and 9 is a new finding as they were not observed on the 51 strains previously described by Yaya et al. (2008) neither in work done by Nwankpa (2008) in Northern Nigeria. Consequently, a new allele number (allele numbers 8 and 9) was given to them.

MLSA allelic sequence comparison of field Isolates with the vaccine reference strain (T1/44).

After alignment of the twenty one field isolates sequences with the vaccine reference strain sequence (T1/44), points of mutation were observed at two positions: positions 1525 and 1750 (Figure 5). Isolates A3 and A8 had A instead of T at position 1525 and 1750 respectively. All other isolates were identical with the vaccine (T1/44) at Loc-PG-0001 of the PG 1 reference strain (Figure 6).

Genbank Accession Numbers

These twenty one sequence data have been submitted to

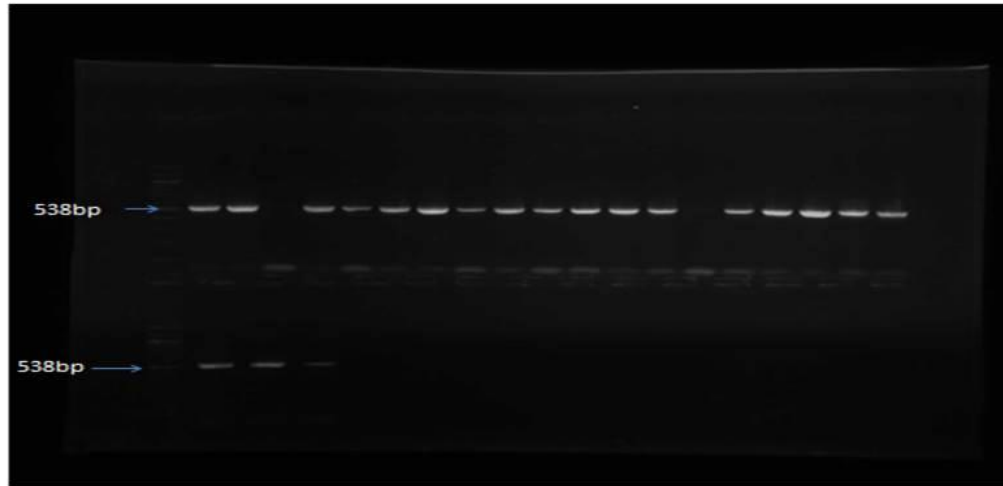


Figure 2. PCR confirmed isolates prior to sequencing with the expected band size of 538bp for the locus: LocPG-0001.

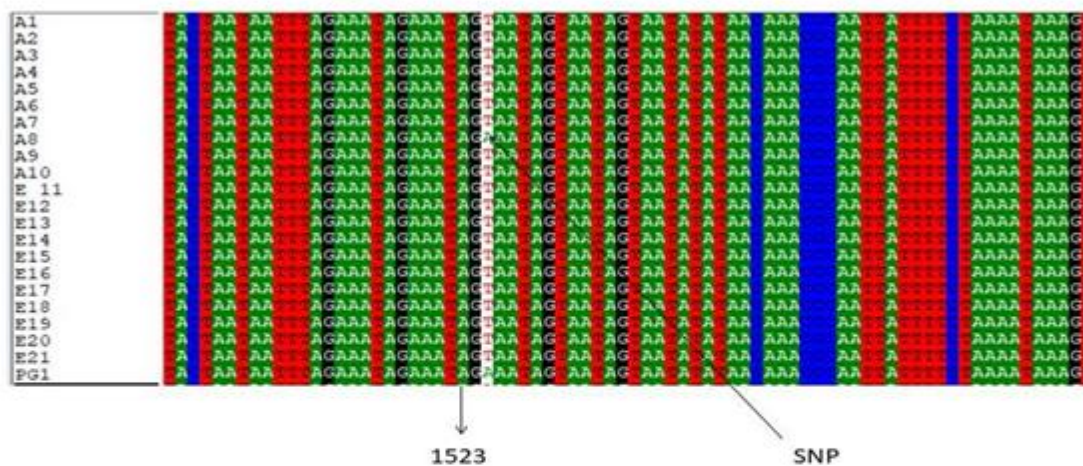


Figure 3. Alignment of sequences on locus Loc-PG1-0001; polymorphism at position 1525 of PG1 reference strain. Isolate A8 had A while the other isolates had T. Key: SNP: single nucleotide polymorphism.

the DDBJ/ Genbank database under accession numbers; MW487814-MW487834. Addresses are as follows:

DNA Data Bank of Japan (DDBJ)
<http://www.ddbj.nig.ac.jp>
 GenBank: <http://www.ncbi.nlm.nih.gov>

DISCUSSION

Contagious bovine pleuropneumonia is a trans-boundary animal disease, with economic effects estimated at billions of Naira in Nigeria (Fadiga et al., 2013). Since the introduction of the disease in Nigeria, its control has

faced many challenges, especially due to lack of efficient data. Countries that have successfully eradicated CBPP relied heavily on data and other programmes such as stamping out policy with commiserate compensation of farmers (Egwu et al., 1996, Thiaucourt et al., 2006). Control of this disease has become a challenge in Nigeria. Studies have documented an upsurge in CBPP outbreaks in South Eastern region of Nigeria mainly due to transhumance from the northern part of the country (Anyika et al., 2021).

This work reports the first molecular analysis of *Mmm* isolated from south east, Nigeria and comparison with vaccine reference strain T1/44. The use of molecular biology tools has greatly improved the capability to

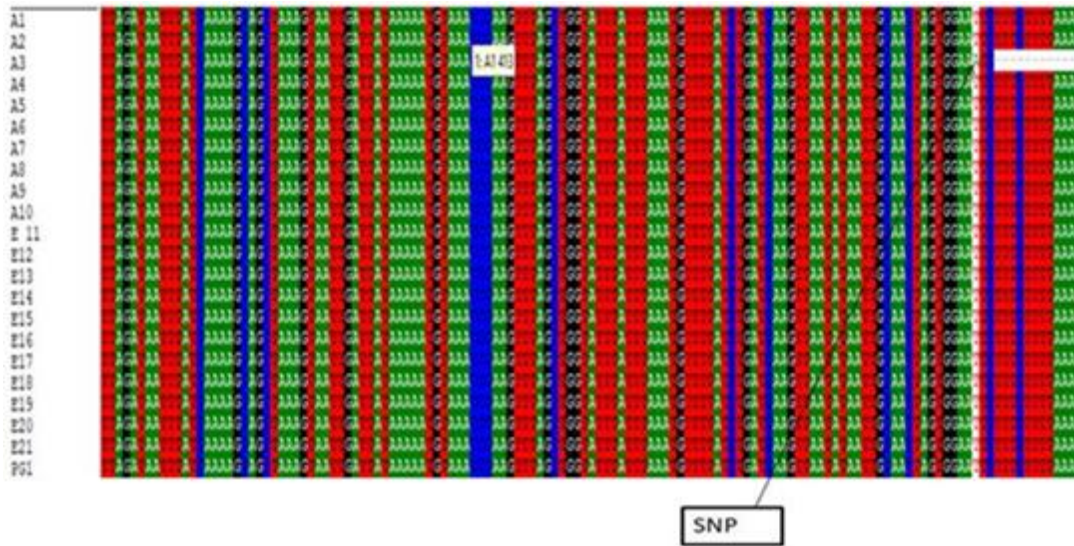


Figure 4. Sequences alignment on locus Loc-PG1-0001; polymorphism at position 1751 of PG1 reference strain. Isolate A3 had A while the other isolates had T. Key:SNP: single nucleotide polymorphism.

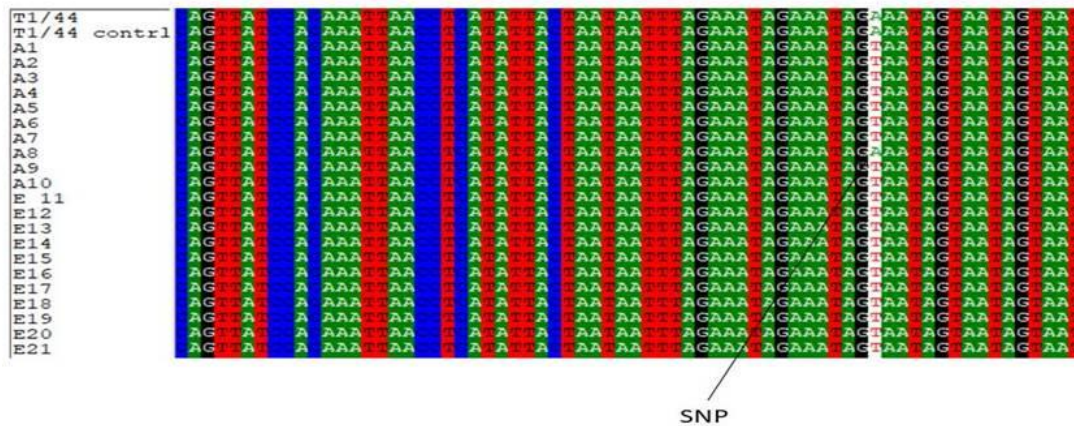


Figure 5. Sequence alignment on the vaccine reference strain (T1/44); polymorphism at position 1525 of T1/44 reference strain. Isolate A8 had A while the other isolates had T. Key:SNP: single nucleotide polymorphism.

detect, identify and characterize strains (Bashiruddin et al., 1994; Thiaucourt et al., 2000a). Three allelic numbers were observed in this study (Allele 1, 8 and 9). Allele 1 was found in both Anambra state and Enugu state while Allele number 8 and 9 were identified only in Anambra State. Allele 1 was earlier reported in the study by Nwankpa (2008) in Northern Nigeria. According to his work, it was found circulating in cattle in both Kaduna and Taraba State of Nigeria. This could be possible, as most cattle slaughtered in south eastern region of Nigeria are bought and transported from the Northern parts of the country. There is a large consumption of beef in south east Nigeria. This could also be the reason why Allele 1

earlier identified by Nwankpa (2008) in Northern Nigeria was also found in the south eastern region. However, Allele 8 and 9 are new strains of *Mmm* that have not been earlier reported in both the works of Yaya et al. (2008) and Nwankpa (2008). This could be a new strain of *Mmm* circulating within the south eastern region of Nigeria. It is however, important to extend this study to other parts of Nigeria to determine if there are such similar *Mmm* profiles in other regions of the country. The new Allele numbers (1, 8 and 9) identified in this study, may be under profile A strains according to groupings by Yaya et al. (2008), which established thirty one allelic profile using eight loci. These profiles are divided into

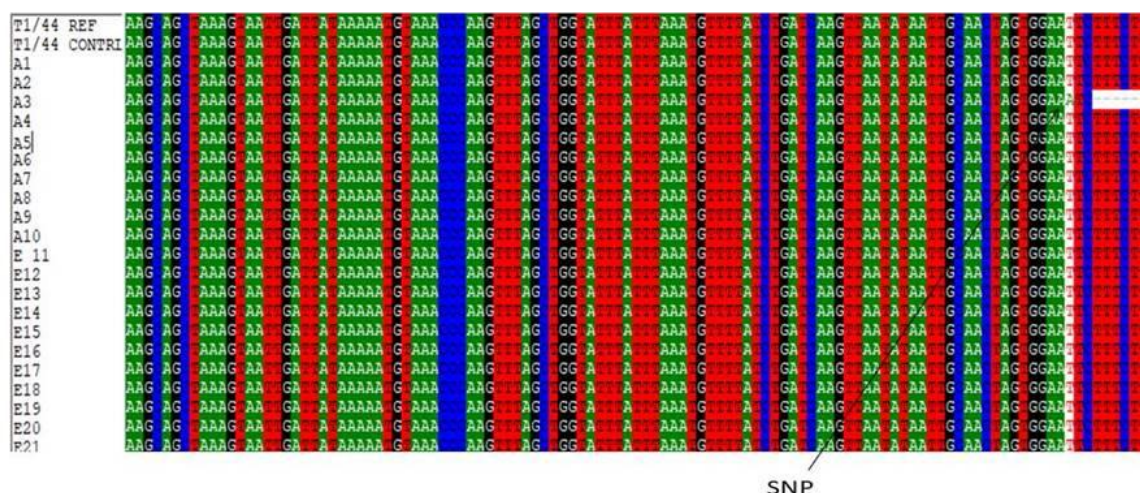


Figure 6. Sequence alignment of vaccine strain T1/44; polymorphism at position 1750 of T1/44 reference strain. Isolate A3 had A while the other isolates had T.
Key: SNP: Single nucleotide polymorphism.
Genbank Accession Numbers.

seven groups: A, B, C, D, E, F and G. Profile A had the largest number of strains with thirteen profiles and are from African origin especially from West Africa. Similarly, Mahamadou et al. (2021) also identified strains under profile A with a novel profile A15 in Niger. Finally, the determination of two new allele numbers not described in previous works of Nwankpa (2008) in Northern Nigeria, is a significant finding in this study.

There are several vaccine strains of CBPP; KH3J, T1SR and T1/44 (Aliyu et al., 2003; Egwu et al., 1996). Presently, T1/44 vaccine strain is used in Nigeria for the control of CBPP (Aliyu et al., 2003). In this study, points of mutation were observed between the field isolates and the vaccine reference strain (T1/44). Perhaps, these observed differences (polymorphism) may account for the low effectiveness of the vaccine (T1/44) to fully protect cattle from field infections as reported by several authors (Thiaucourt et al., 2000a; Thiaucourt et al., 2006; Yaya et al., 1999). For instance, isolate A3 from Anambra state had A at position 1560 of the T1/44 reference strain while other isolates had T. Perhaps, this change in Nucleotide sequence, could account for failure of the vaccine to confer maximum immunity against CBPP. However, more studies need to be carried out to ascertain these claims. Several studies have reported similar differences between the field isolates of CBPP in Nigeria with the Vaccine strain. Folashade (2014) reported difference between the vaccine strain and field strain using restriction fragment length polymorphism. Similarly, Nwankpa (2008) also reported differences between the sequences of the field isolates from some Northern states of Nigeria and the vaccine strain (T1/44), he observed points of polymorphism among some of the field isolates from Northern Nigeria and the vaccine strain (T1/44).

The efficacy of CBPP vaccine in protecting cattle against infection has been widely studied (Thiaucourt et al., 2000a; Wesonga and Thiaucourt, 2000; Yaya et al., 1999). Some of these studies have indicated the inability of the vaccine to effectively protect cattle against contagious bovine pleuropneumonia (March and Brodie, 2000). This underscores the need for newer approach in the control of CBPP in Africa. Perhaps, there is the need to develop a polyvalent CBPP vaccine, which will contain two or more different strains of *M. mycoides* Subsp. *mycoides* alongside the T1/44 currently used. We believe that a better CBPP vaccine would increase the chances of eradicating the disease from the continent.

Conclusion

This study identified two novel alleles: Allele 8 and 9 and observed sequence differences between the field isolates of *M. mycoides mycoides* and the vaccine reference strain (T1/44) in south east Nigeria.

RECOMMENDATION

This molecular method should be extended to other parts of the country, to effectively characterize other circulating strains of *Mmm*. This should improve the surveillance and control of this disease in Nigeria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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