Molecular detection of Nigerian field isolates of *Mycoplasma mycoides* subsp. *mycoides* as causative agents of contagious bovine pleuropneumonia

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**Abstract** Contagious bovine pleuropneumonia (CBPP) is a highly contagious respiratory disease affecting cattle and is widely distributed in the sub-Saharan Africa. The objective of this study was to detect *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) the causative agent of CBPP from 90 cattle at slaughter using polymerase chain reaction-Restriction fragment length polymorphism. In this study, 450 samples suggestive of CBPP in Maiduguri, Yola and Gombe township abattoirs were processed according to standard protocols. The isolation rate was found to be 3.33% and percentage of identification with PCR-RFLP yielded 1.56%. Subsequently, QIAxcel revealed molecular size of 574 bp for *Mycoplasma mycoides* subcluster. Further analysis of PCR amplicons with restriction digestion, confirmed the presence of *Mmm* 16S rRNA of CAP 21 genomic region with molecular sizes of 180 bp and 380 bp. Thus, the 380 bp fragments delineated *Mmm* from *Mycoplasma mycoides* subsp. *capri*. Three isolates (BL5, BL6 and AL1) were from lungs and four from pleural fluids (APF2, APF8A, APF8B and APF9) were isolated and identified, while a vaccine strain T1/44 was re-detected along with the field isolates. No sample from Gombe had *Mmm*. In conclusion, the findings of this study have detected the presence of *Mmm* as causative agent of CBPP. Measures such as surveillance, quarantine and vaccination are hereby recommended for the control of CBPP in Nigeria.

**1. Introduction**

*Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) is the causative agent of contagious bovine pleuropneumonia (CBPP), a severe, contagious respiratory disease affecting cattle and is...
widely distributed in Africa [1–3]. It is included among the Office International des Epizooties (OIE) diseases requiring official notification leading to banning of countries involved from participation in international cattle trade [4]. CBPP has continued to exist as an endemic disease in several countries in the sub-Saharan Africa [5].

CBPP could be manifested in hyperacute form, which may be rapidly fatal with no clinical signs observed, while the subacute form is frequent, characterized by mild or no clinical signs at all [6]. It is known that animals with these forms are able to transmit the infection to other cattle in the herd [7]. The acute form is characterized by increase in body temperature, moderate respiratory distress and painful and irregular cough, abstraction of the forelimbs, extension of the neck, arched back and dilatation of the nostrils [6,7]. In chronic form, the animal may remain in poor condition for a long period, depending on the size of the chronic lung lesion. Fever is intermittent and the animals with sequestra may be normal in appearance while harbouring bacteria [6,8]. Several countries have eradicated CBPP, but the disease still remains a serious problem in certain parts of Africa, which includes Nigeria where CBPP is endemic [9–14].

The causative agent of CBPP are presumptively identified as comet in broth and ‘fried eggs’ colonial appearance on the pleuropneumonia like-organism (PPLO) agar media [15,16]. Additionally, molecular typing could identify Mycoplasma mycoides subcluster and Mmm at the molecular sizes of 574 bp as well as 180 bp and 380 bp respectively [17–19].

Currently the control of CBPP in sub-Saharan Africa is largely dependent on a live vaccine, T144 [20,21]. The vaccine has low efficacy, limited duration of immunity, sometimes severe side effects and require cold chain to maintain its potency [21]. The low efficacy of the current live vaccine, together with the costs of vaccination campaign remains a serious challenge for the African countries in the control of CBPP [22–24]. After primary vaccination protection coverage may be as low as 30–60% [25].

The strains used in this study were isolated from abattoir field samples. Mycoplasmas were grown at 37 °C under 5% CO2 in PPLO broth and agar (Difco) supplemented as previously described [26,27]. The samples were processed in OIE Reference Laboratory for CBPP – Istituto Zooprofilattico Sperimentale dell’Abruzzo e Molise (IZSAM) ‘G. Caporale’, Campo Boario Teramo, Italy. On receiving the samples, they were stored at −20 °C until required. After the arrival at the OIE Reference laboratory, the samples were removed, verified and aseptically packaged and then registered under exotic diseases unit prior to further processing.

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2.3. Isolation and growth condition of mycoplasmas

Deoxyribonucleic acid (DNA) was extracted from 1000 μl of culture using commercially available Maxwell® 16 Tissue/cell DNA Purification Kits (Promega, USA) with Maxwell® 16 instrument (Promega, USA) according to the manufacturer’s instructions. Briefly, the culture in the Eppendorf tubes was centrifuged at 1600g/10 min. The supernatant was discarded and the pellets diluted with 300 μl of lysis buffer in Eppendorf tubes and then pipetted into the first chamber of the DNA purification cartridge. The DNA purification kit was inserted into the Maxwell® 16 instrument and then run for 36 min.

2.4. Molecular detection of Mycoplasma mycoides subcluster

2.4.1. Extraction of genomic DNA

The protocol was adapted from the OIE Reference Laboratory for CBPP and a PCR-based test for the specific identification of Mycoplasma mycoides subspecies mycoides SC from [17]. Specific primers were used to amplify one band of 574 bp expected fragment for Mycoplasma mycoides subcluster (Mmm and Mmc). The forward and reverse primers were designed from CAP-21 genomic region.

MM450: 5'-GTATTTTTCCITTACTTTTG-3' MM451: 5'-AAATCAATTATAAGTTTG-3' The 50 μl volume was required: 25 μl of Top Taq Master Mix containing; Taq Polymerase, dNTPs, MgCl2 and Taq buffer, 18 μl nuclease free water, 1 μl forward primer, 1 μl reverse primer and 5 μl of genomic DNA.

The amplification reaction was carried out under the following conditions: initial denaturation temperature at 94 °C
for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 5 min and standby at 4 °C in GeneAmp® PCR System, 9700 (Applied Biosystems). *Mycoplasma mycoides* subspp. *mycoides* and *Mmc* were used as positive controls.

**Table 1** Profile of isolates of *Mycoplasma* and *Mycoplasma mycoides* subspecies *mycoides* confirmed with polymerase chain reaction-Restriction fragment length polymorphism.

| Sample source           | Sample type    | No. of samples collected | No. of *Mycoplasma* grown on agar (%) | No. of positives strains with PCR-RFLP (%) |
|-------------------------|----------------|--------------------------|---------------------------------------|-------------------------------------------|
| Maiduguri-Borno State   | Lungs          | 30                       | 2 (6.67)                              | 2 (6.67)                                  |
|                         | Lymph nodes    | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Pleural swabs  | 30                       | 1 (3.33)                              | 0 (0.00)                                  |
|                         | Ear swabs      | 30                       | 2 (6.67)                              | 0 (0.00)                                  |
|                         | Nasal swabs    | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Lungs          | 30                       | 2 (6.67)                              | 1 (3.33)                                  |
|                         | Lymph nodes    | 30                       | 3 (10.00)                             | 0 (0.00)                                  |
|                         | Pleural swabs  | 20                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Pleural fluids | 10                       | 4 (40.00)                             | 4 (40.0)                                  |
|                         | Ear swabs      | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Nasal swabs    | 30                       | 1 (3.33)                              | 0 (0.00)                                  |
| Yola-Adamawa State      | Lungs          | 30                       | 2 (6.67)                              | 1 (3.33)                                  |
|                         | Lymph nodes    | 30                       | 3 (10.00)                             | 0 (0.00)                                  |
|                         | Pleural swabs  | 20                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Pleural fluids | 10                       | 4 (40.00)                             | 4 (40.0)                                  |
|                         | Ear swabs      | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Nasal swabs    | 30                       | 1 (3.33)                              | 0 (0.00)                                  |
| Gombe-Gombe State       | Lungs          | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Lymph nodes    | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Pleural swabs  | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Ear swabs      | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
|                         | Nasal swabs    | 30                       | 0 (0.00)                              | 0 (0.00)                                  |
| NVRI-Vom, Jos Plateau State | T1/44 Vaccine vial | 1 (100.00)           | 1 (100.00)                            | 1 (100.00)                                |

Fig. 1 Distribution of sampling location in North-Eastern Nigeria. Source: Designed by GIS Unit IZS, Teramo Italy.
Polymerase chain reaction products were analyzed by capillary electrophoresis using QIAxcel-Pure Excellence (Qiagen). The technique was based on a DNA size marker or molecular marker set between 100 bp and 2500 bp, which was run with the 7 isolates of *Mycoplasma* (found to express fried eggs appearance on PPLO agar) and T1/44 in 96 wells plate. Pre-programmed marker table allowed the assigning of the electrophoresed DNA, the known size in bp to the detected peaks. Based on the bp/migration time ratio of the DNA size marker the software calculated the size of all unknown fragments.

### 2.6. PCR-RFLP for Mycoplasma mycoides subsp. mycoides

The typing method was adapted from the protocol used in OIE Reference Laboratory for CBPP and that of [18], but varied with respect to *Vsp1* (8–12 U/μL concentration) which replaced *Asn1* endonuclease in this study.

The master mix comprised of; 0.4 μl of restriction endonuclease (*Vsp1*), 3.6 μl of nuclease free water, 1 μl of specific buffer and 5 μl of the PCR product been a preparation for a single sample. The mixture was centrifuged at 16,875 g/45 s and then incubated at 37 °C for 2 h. Polymerase chain reaction products were digested with restriction enzyme *Vsp1*. The products were separated by electrophoresis (agarose × 3% in TBE buffer) at 100 volts for a period of 20 min. At the end of the run, the resulting DNA fragments were analyzed by visualization under UV light to determine the expected base pairs of the fragments.

### 3. Results

#### 3.1. Profile of samples for *Mycoplasma* and *Mycoplasma mycoides* subsp. *mycoides* isolates identified on PPLO agar media

Out of the total of 450 samples inoculated onto PPLO agar media, 2 (6.67%) lungs samples, 1 (3.33%) pleural fluids and 2 (6.67%) ear swab samples from Maiduguri, gave typical fried eggs colonies. Two (6.67%) lung samples, 3 (10.00%) lymph nodes, 4 (40.00%) pleural fluids and 1 (3.33%) nasal swab from Yola, yielded characteristic growth of *Mycoplasma*. The isolation rate on PPLO agar media was found to be 15 (3.33%). No sample gave typical colonies from Gombe (Table 1).

#### 3.1.1. Lung sample showing CBPP lesion

The marbling appearance of the lung characterized by pneumonia and pleurisy, thickened interlobular septae and hepatization suggestive of CBPP is shown in (Fig. 2).

#### 3.1.2. Colony morphology for *Mycoplasma*

The colonies of *Mycoplasma* showing typical fried eggs morphology were observed. The fried egg colony is labelled a, while b indicated the dense centre. The classical colonies were recorded at ten days of incubation (Fig. 3).

#### 3.2. QIAxcel capillary electrophoresis for *Mycoplasma mycoides* subcluster

The QIAxcel capillary electrophoresis of PCR amplicons for *Mycoplasma mycoides* subcluster is shown in (Fig. 4). Lanes O1A to O7B are the samples in duplicate (50 μg and 30 μg concentration), lanes O8A to O8B is T1/44, followed by positive controls for *Mmm* and *Mmc* on lanes O9A and O9B and a negative control on lane O10A. The standard molecular marker

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**Fig. 2** A typical CBPP infected cattle lung cut open to reveal the marbled appearance from Maiduguri abattoir, North-Eastern Nigeria. Pneumonia and pleurisy (a), hepatization (b), thickened interlobular septae (c).

**Fig. 3** *Mycoplasma* isolated from Yola at ten days of incubation (25X) at IZS, Teramo, Italy.
ranged from 100 bp to 2500 bp and the alignment marker is located between 15 bp and 3000 bp. The samples of *Mycoplasma mycoides* subcluster presented fragment sizes of 574 bp.

### 3.3. Percentage PCR-RFLP for *Mycoplasma mycoides* subspecies *mycoides*

Polymerase chain reaction-RFLP gave 2 (100.00%) from Maiduguri lungs, 1 (50.00%) and 4 (100.00%) from lung and pleural fluids in Yola. The percentage of identification by PCR-RFLP was 7 (1.56%). Seven out of the fifteen *Mycoplasma* isolates identified in cattle at slaughter; three samples from lungs and four from pleural fluids were confirmed as *Mmm* with PCR-RFLP. T1/44 was re-confirmed as *Mmm* (Table 1).

### 3.4. PCR-RFLP amplicons on 3% gel electrophoresis identified as *Mycoplasma mycoides* subsp. *mycoides*

Seven *Mycoplasma* isolates and T1/44 (in duplicate) were identified as specific fragments. The PCR-RFLP amplicons on 3% gel electrophoresis for the identification of *Mycoplasma mycoides* subspecies *mycoides* has been presented. Lanes 1 and 20 are the molecular markers, lanes 2 to 3 are...
samples from Maiduguri (in duplicate), lanes 4 to 16 are samples from Yola, lane 17 is T1/44, and lanes 18 and 19 are \textit{Mmm} and \textit{Mmc} positive controls. Two different molecular sizes of 180 bp and 380 bp specific for \textit{Mmm} was revealed by PCR-RFLP amplicons on 3% agarose gel. No band yielded 150 bp, 180 bp and 230 bp except the positive control for \textit{Mmc}. The 380 bp fragment delineated \textit{Mmm} from \textit{Mmc} (Fig. 5).

4. Discussion

This study has demonstrated that cattle in Maiduguri and Yola harbour \textit{Mmm} the causative agent of a highly contagious trans-boundary disease-CBPP. The isolation and molecular technique, PCR-RFLP provided means for specific identification of \textit{Mmm} from slaughtered cattle in Northeastern zone of Nigeria. Although CBPP like lesions were sampled from all the three abattoirs, no \textit{Mmm} was identified in samples obtained in Gombe abattoir. The identification of the causative agents of CBPP is in agreement with earlier report [11], who stated that CBPP is endemic in Borno (remained as Borno) and Gongola (now Adamawa). Additionally, an abattoir based study equally reported a lesion based prevalence of 0.29% in five States of Northern Nigeria [13]. The reason for the absence of \textit{Mmm} isolates from Gombe abattoir, may not be unconnected with the fact that the disease might be absent in those animals at the time of sampling. The presence of antibiotics might have contributed to failure to recover the mycoplasmas and the CBPP-like lesions might have been mistaken for other causes of pleuropneumonia, such as \textit{Pasteurella} species and other pathogens as earlier reported [27].

The small colonies with the classical ‘fried-eggs’ appearances of \textit{Mycoplasma} on the agar media were in consonance with the report of [15]. Two of the strains (APF8A and APF8B) derived from APF8, which presented two different colonies on the agar media were presumptively identified as \textit{Mycoplasma} in this study. The APF8A displayed typical fried egg colony, while APF8B failed to give the typical colony of \textit{Mycoplasma}. The reason for this occurrence remained to be determined.

The isolation rate of 3.33% found in this study was lower than 6.9% reported in pneumonic cattle from Bosnia and Herzegovina [28]. The identification rate of \textit{Mmm} in this study was found to be 1.56%, higher than 0.00% reported by [27] who could not isolate \textit{Mmm} from cattle at slaughter in an abattoir study conducted in Turkey.

The \textit{Mycoplasma} isolates found in this study were isolated from consolidated lungs, pleural fluids that were suggestive of CBPP. The 7 isolates confirmed as \textit{Mmm} were from lungs (BL5, BL6 and AL1) and pleural fluids (APF2, APF8A, APF8B, APF9). This could be explained by the fact that the main predilection site for \textit{Mmm} is the lung where \textit{Mmm} causes pathological lesions accompanied with the production of pleural fluids- thus, creating a more conducive environment for the survival of \textit{Mmm}.

The PCR amplification specific for \textit{Mycoplasma mycoides} subcluster was found to be positive for all the 7 isolates and T1/44 which yielded molecular size of 574 bp specific for \textit{Mmm} or \textit{Mmc}. The identification of \textit{Mycoplasma mycoides} subcluster at molecular size of 574 bp is in agreement with the findings of [18,19]. The molecular size of 574 bp is unable to distinguish between the two members of the \textit{Mycoides} sub-cluster; \textit{Mmm} which causes CBPP in cattle and \textit{Mmc} which affects mostly small ruminants where it induce lesions such as mastitis, arthritis, kerato conjunctivitis, pneumonia and septicaemia [29].

The PCR-RFLP identified the 16S rRNA for CAP-21 genomic regions of 7 isolates of \textit{Mmm} the causative agent of CBPP, and re-confirmed T1/44 at the molecular sizes of 180 bp and 380 bp. Furthermore, the 380 bp delineated \textit{Mmm} from \textit{Mmc}. This finding is in consonance with the work of [18,19]. These further confirmed that strains of \textit{Mmm} are
circulating in cattle in Maiduguri and Yola, North-eastern zone of Nigeria. The PCR-RFLP based on restriction endonuclease activity, could discriminate between isolates of *M. mycoides* and *Mmc* isolates. This method could be a useful tool to distinguish between the *Mycoplasma* isolates and for strain typing to enable detection of *Mmm* [17,18].

5. Conclusion

The findings of this study have detected the presence of *Mmm* as causative agent of CBPP. The PCR-RFLP is an important technique for the detection of *Mmm*. The lung lesions and pleural fluids are the most probable samples from which *Mmm* can be detected in cattle. We therefore recommend measures such as targeted abattoir surveillance, quarantine and vaccination for the control of CBPP in Nigeria.

Competing interests

The authors declare that they have no competing interests.

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