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Molecular epidemiology of contagious bovine pleuropneumonia by detection, identification and differentiation of *Mycoplasma mycoides* subsp. *mycoides* in Niger State, Nigeria

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Abstract

The study was aimed at epidemiologically detecting, identifying, and differentiating *Mycoplasma mycoides* subsp. *mycoides* strains according to their geographical origins in asymptomatic cattle populations of Niger State, North-central Nigeria between January and August 2013, using conventional and fingerprinting PCR assays without culturing or enriching of the field samples. Cross sectional molecular study of asymptomatic cattle sampled in the major abattoirs and livestock markets was carried out. Geographical lineage differentiation of the strains was performed on the basis of presence or absence of 8.84 kb genomic segment. With primers pair MM450/MM451, two 574 bp genomic fragments of DNA of *Mycoplasma mycoides* subsp. *mycoides* were detected. Using fingerprinting PCR; with insertion element IS1296/R(all) primers pair the detected two strains of *Mycoplasma mycoides* subsp. *mycoides* genomic DNA were specifically identified by generation of a fragment of 1.1 kb; with IS1296/R1 primers pair, identified *Mycoplasma mycoides* subsp. *mycoides* strains were differentiated to be of African cluster by generation of 1.4 kb. The PCR procedures were sensitive and produced rapid results that identified two strains of *Mycoplasma mycoides* subsp. *mycoides* of African/Australian origin. The presence of *Mycoplasma mycoides* subsp. *mycoides African*/Australian strains, which is useful for vaccine production, was therefore established in Niger State, Nigeria.

Keywords: Cattle, Control, Cross-sectional molecular study, *Mmm* , Molecular fingerprinting, Nigeria
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Introduction

Cattle are the most important livestock species, in terms of animal protein supply and economic value in Nigeria and are most commonly found in the northern parts of the country (Bourn, 1992). However, the major militating factor against cattle production in Nigeria is diseases, such as contagious bovine pleuropneumonia (Aliyu *et al.*, 2000). The disease is also a major constraint to cattle production in sub-Saharan Africa (Lorenzon *et al.,* 2003).

Contagious bovine pleuropneumonia (CBPP), caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) previously further specified as Small Colony (SC) type (Manso-Silván *et al.*, 2009), is manifested by anorexia, fever, dyspnoea, polypnoea, cough and nasal discharges (Radostits *et al.,* 2007). The disease was eradicated from the United States and Europe in the early 20th century, though some of them have experienced sporadic outbreaks with affected herds manifesting little distinctive clinical signs and lower mortality than those found in Africa

(Nicholas *et al.,* 1996; Regalla *et al*., 1996; Nicholas *et al.,* 2008). On all occasions, field epidemiological inquiries failed to yield clues regarding the origin of the outbreaks and did not enable the elucidation of whether they were the result of reintroduction or resurgence of the disease (Yaya *et al*., 2008).

Phylogenetically, *Mmm* is a member of the *Mycoplasma mycoides* cluster, which has six members that are pathogens of small and large ruminants (Manso-Silvan *et al.,* 2009). Identification of members of this group is associated with difficulties due to shared biochemical, immunologic, and genetic characteristics (Miles *et al.,* 2006). Although *Mmm* is thought to be a very homogeneous taxon, previous studies have shown *Mmm* strains are geographically heterogeneous which could be differentiated using molecular typing assays and grouped into two major epidemiological and clinical distinct clusters: one cluster contains isolates from different European countries and a second cluster contains Africa and Australian strains (Cheng *et al*., 1995; Lorenzon *et al.,* 2003; Yaya *et al*., 2008). Genetic analysis of representative strains from the clusters revealed a genomic segment of 8.84 kb, which is present in all strains of the Africa and Australian cluster but lacking in all strains of the European cluster, with African and Australian strains potentially more virulent than the European strains (Houshaymi *et al.,* 1997). Parts of the challenges of CBPP control have been the difficulty in disease diagnosis, which may be attributable to the fact that majority of animals infected with the disease do not show clinical signs at chronic stages and this poses a major problem in surveillance (OIE, 2010) and availability of different geographical lineages of *Mmm*.

CBPP is usually confirmed by culture and serology, but mycoplasmas are slow growing and culture can take up to three weeks for isolation and identification. In individual live cattle, confirmation of CBPP is problematic because of low specificity of the existing serological tests, such as the complement fixations test (CFT) and competitive enzyme linked immune-sorbent assay (c-ELISA), to address issues of cross-reactions among closely related members of *M. mycoides* cluster under epidemiological conditions. These are only useful as herd tests for the detection of natural infections and not for individual infected animals (Enyaru *et al.,* 2012).

However, polymerase chain reactions (PCR) can detect *Mmm* strains in blood, tissues and body fluids of individual cattle, even in chronic ones (Schnee *et al.,* 2011), as well as differentiating these strains according to their geographical lineages (Poumarat & Solsona, 1995; Miles *et al.,* 2006). A precise differentiation of *Mmm* must be established for control strategies since virulence consequences differ widely depending on the clusters.

This study was aimed at epidemiologically detecting and identifying *Mmm* strains under field conditions in asymptomatic pastoral cattle of Niger State, North-central Nigeria, and differentiating them according to their geographical origins for active surveillance and effective control. To ensure the robust and sensitive identification of *Mmm* strains and their geographical clusters, we have modified two PCR assays of conventional PCR and fingerprinting that target *Mmm* strains genes.

Materials and methods

Study area

The study was conducted in Niger State located at the North-central geopolitical zone of Nigeria, between latitude 8^{o} 20' N and 11^{o} 30' N, and longitude 3^{o} 30'E and 7^{o} 20'E. It provides transit routes for pastoral nomads on seasonal migrations from the northern parts to the southern areas and back, and shares an international boundary with the Republic of Benin at its western border. The state has an estimated cattle population of 2.4 million cattle under the custodies of pastoralists (MLFD, 2013).

Study design and populations

Cross sectional molecular study of asymptomatic cattle sampled in major abattoirs (Minna, Suleja, Bida, Kontagora, and New-Bussa) and livestock markets (Jebba, Beji, Mariga, Tungan-mallam, Kuta, and Lambatta) in the state between January and August, 2013.

Sample size and sampling method

The sample size was determined using random method (Thrusfield, 2009) and CBPP expected prevalence of 8.7% (Alhaji, 2011) at 95% confidence level. Sample size was determined at 5% margin of error and 125 cattle were obtained. Purposive sampling was carried out.

Samples collection

Two approaches were used: (i) Blood sample; 10ml of blood was collected from jugular veins of the selected 62 asymptomatic cattle into EDTA bottles using sterile 10ml syringes and 18 x 1½ gauge needles at the major livestock markets in the state.

(ii) Lung tissues, mediastinal lymph nodes, 5ml of pleural fluid (from one animal) were collected at postmortem from 63 selected asymptomatic cattle earlier inspected before slaughtered at the major slaughterhouses in the state.

All samples were preserved at -20^{o}C in the Niger State Veterinary Hospital, Minna laboratory before being transported to the Applied Molecular Biology Division, National Veterinary Research Institute (NVRI), Vom, Nigeria for DNA extractions and molecular analysis. All samples were not subjected to enrichment or culturing before DNA extractions.

DNA extractions

The lung tissue and lymph node samples were minced into small pieces, pulverized and transferred into 2ml microfuge tubes and homogenized in phosphate-buffered saline (PBS) solution. ZR Fungal/Bacterial DNA MiniPrep™ commercial extraction kits (Zymo Research Corporation, USA) were used for extractions of bacterial genomic DNA present in the field samples according to manufacturer's instructions. The resulting ultra-pure bacterial DNA was obtained and stored at -20^{o}C for use in molecular analyses.

Determination of field samples which yield bacterial DNA

In determining the DNA yields of the 125 field samples, the extracts were all subjected to 2% w/v agarose gel electrophoresis stained with ethidium bromide dye at 80 voltages for one hour. Samples with bacterial DNA were imaged under the ultraviolet transilluminator of Bio-Rad Molecular Imager® (Bio-Rad Laboratories Inc., USA).

Molecular detection of Mmm

In this PCR assay, a modified version of previously published protocol (Bashiruddin *et al*., 1994; Solano *et al.,* 2005; Enyaru *et al.,* 2012) using primer pair MM450 and MM451 (Table 1) was conducted to detect *Mmm* strains. DNA amplification was performed using 5 µl of test DNA template in a total volume of 50 µl for 40 cycles. The reaction mixture consisted of 10 mM Tris-HC1 (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 µM dNTP mix and 1.5 U of DreamTaq™ Green DNA Polymerase (Thermo Scientific, USA). Each primer was used at 50 pmoles total per reaction. The optimal amplification cycle for both primer sets consisted of denaturation at 94^{o}C for 1 min, annealing at 50^{o}C for 1 min, and extension at 72^{o}C for 2 min and was performed in a GeneAmp® PCR System 9700 version 3.12 Thermal Cycler (Applied Biosystems, USA). The positive control used was provided by the Mycoplasma Research Laboratory of National Veterinary Research Institute, Vom, while nuclease free water (Promega, Madison, USA) was used as negative control. Twenty microliters of each PCR product was then subjected to electrophoresis in a 2.0% w/v agarose gel containing ethidium bromide at 80 voltages for one hour. The amplified products were visualized in positive samples by ultraviolet transilluminator, Bio-Rad Molecular Imager® (Bio-Rad Laboratories Inc., USA).

Specific molecular identification and differentiation

Another modified version of previously published PCR assay (Miles *et al.,* 2006) was carried out. It was used to specifically identify and differentiate *Mmm* strains DNA genomes according to their geographical origins using insertion sequence element fingerprinting. This assay has three protocols:

Protocol 1 – This test was used to specifically amplified *Mmm* DNA using oligonucleotides pair IS*1296* and R(all) (Table 1), with both of them inserted upstream of the deletion breakpoint. Amplification was performed using: 0.4 µM insertion element (IS1*296*) and 0.4 µM R(all) oligonucleotides. All reactions were carried out in a final volume of 50 µl and contained 15 mM Tris-HCl, pH 8.0; 50 mM KCl; 2.0 mM $MgCl_2$; 400 mM dNTP mix, and 2.5 U of DreamTaq™ Green DNA Polymerase (Thermo Scientific, USA). The samples were subjected to 40 cycles of amplification reactions performed in a GeneAmp^{R} PCR System 9700 version 3.12 Thermal Cycler (Applied Biosystems, USA) with an initial denaturation step of 95^{o}C for 5 min, followed by 32 cycles of denaturation at 95^{o}C for 30 sec; annealing at 62^{o}C for 30 sec; and extension at 72^{o}C for 1 min 20 sec. A final extension at 72^{o}C for 5 min was included.

Protocol 2 - Differentiation of *Mmm* strains as African/Australian geographical origin. This was conducted using primers pair IS*1296* and R1 (Table 1). The insertion element IS*1296* was inserted upstream of the 8.84 kb deletion of the bacterial deletion region and R1 oligonucleotide was designed to be within the deletion point to produce fragments only of African/Australian origins. All reactions were carried out in a final volume of 50 µl, and contained 15 mM Tris-HCl, pH 8.0; 50 mM KCl; 400 mM dNTP mix: 0.4 mM each oligonucleotide, 2.0 mM $MgCl_2$, and 2.5 U of DreamTaq™ Green DNA Polymerase

(Inqaba Biotec). The samples were subjected to 40 cycles of amplification reactions carried out in a GeneAmp® PCR System 9700 version 3.12 Thermal Cycler with an initial denaturation of 95°C for 5 min, followed by 32 cycles of denaturation at 95°C for 30 sec; annealing at 60°C for 30 sec, and extension at 72°C for 1 min 30 sec.

Protocol 3 – This was performed using primers pair IS*1296* and R2. The insertion IS*1296* was inserted upstream of the 8.84 kb of the bacterial deletion. The R2 oligonucleotide was designed to be placed downstream to detect *Mmm* DNA fragments only of European origin. All reactions were carried out in a final volume of 50 µl, and contained 15 mM Tris-HCl, pH 8.0; 50 mM KCl; 400 mM dNTP mix: 0.4 mM each oligonucleotide, 2.0 mM $MgCl_2$, and 2.5 U of DreamTaq™ Green DNA Polymerase (Inqaba Biotec). The samples were subjected to 40 cycles of amplification reactions carried out in a GeneAmp® PCR System 9700 version 3.12 Thermal Cycler with an initial denaturation of 95°C for 5 min, followed by 32 cycles of denaturation at 95°C for 30 sec; annealing at 60°C for 30 sec, and extension at 72°C for 1 min 30 sec.

The positive control used for the three protocols was provided by the Mycoplasmas Research Laboratory of National Veterinary Research Institute, Vom, though its geographical lineage was not known by the provider, while nuclease free water (Promega, Madison, USA) was used as negative control. Twenty microliters of each PCR product was electrophoresed in 2.0% w/v agarose gel containing ethidium bromide at 80 voltages for one hour. The amplified products were visualized by ultraviolet transilluminator, Bio-Rad Molecular Imager®.

Table 1. Sequences of PCR primers used for specific detection, identification and differentiation of *Mycoplasma mycoides* subsp. *mycoides* strains in pastoral cattle of Niger State, Nigeria

S/No	**Primer**	**Sequence (5' to 3')**	**Reference**
1	MM450	GTA TTT TCC TTT CTA ATT TG	Bashiruddin *et al*. (1994); Solano *et al*. (2005); Enyaru *et al*. (2012)
2	MM451	AAA TCA AAT TAA TAA GTT TG	Bashiruddin *et al*., (1994); Solano *et al*. (2005); Enyaru *et al*. (2012)
3	IS1296	CTA AAG AGC TTG GAG TTC AGT G	Miles *et al*., (2006)
4	R(all)	CCA GCT CAA CCA GCT CCAG	Miles *et al*., (2006)
5	R1	GGA AGG TAT GTT TAG CGG AGC	Miles *et al*., (2006)
6	R2	GCA CCA AAT GAA GCT TAA TAG AAC	Miles *et al*., (2006)

Note: All primers were synthesized by Inqaba Biotec™, Pretoria, South Africa

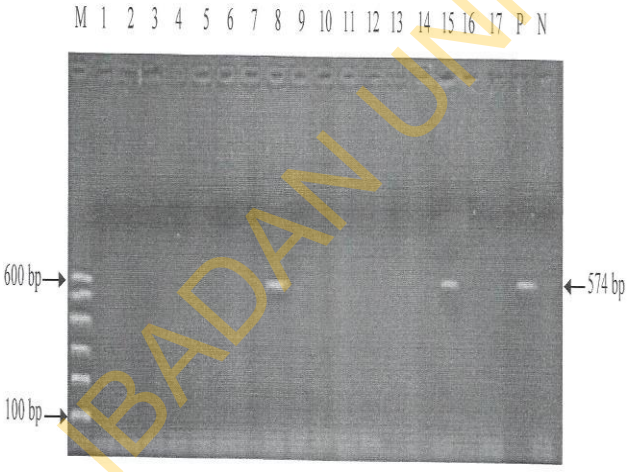

Plate 1. Gel electrophoresis of PCR products of 17 field samples which yielded bacterial genomic DNA. Positive and negative controls in lanes P and N, respectively. Lanes: M = Molecular weight markers (MWM; Bio-Rad 50 – 2000 bp); 8 = *Mmm* at 574 bp; 15 = *Mmm* at 574 bp; and P = positive control (*Mmm*) at 574 bp

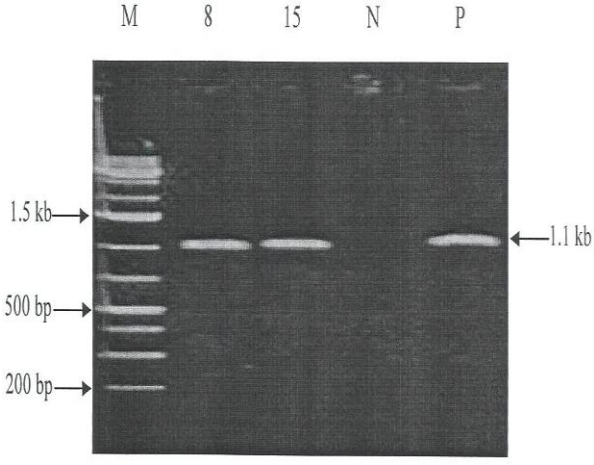

Plate 2. Gel electrophoresis of PCR products of two field samples and controls using primers IS*1296* and R(all). Lanes: M = Molecular weight markers (MWM; Bio-Rad 50 – 2000 bp); 8 = sample 8 *Mmm*; 15 = sample 15 *Mmm*; N = negative control; and P = positive control (*Mmm*)

Statistical analysis

The number of detected, identified and differentiated *Mmm* strains in relation to the total number of filed samples used for the study was analyzed using the Open Source Epidemiologic Statistics for Public Health (OpenEpi) software version 2.3 (Dean *et al.,* 2009).

Results

Detection of Mmm

Of the 125 field samples taken across the state for bacterial DNA extractions, only 17 (13.6%; 95% CI: 8.4-20.5) had bacterial genomic DNA on gel electrophoresis before PCR amplifications. Upon PCR amplifications of the genomic DNA from the 17 field samples and controls using primers pair MM450 and MM451, *Mmm* strains were detected in only two (8 and 15) field samples (1.6%; 95% CI: 0.3-5.2) and positive control, as indicated by amplification of 574 bp fragments on gel (Plate 1).

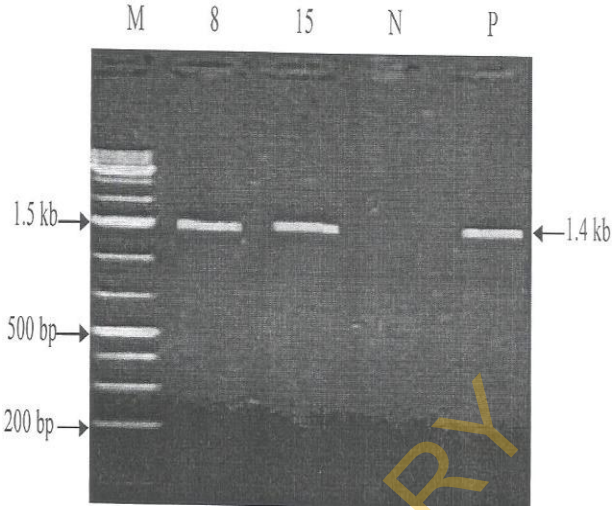

Plate 3. Gel electrophoresis of two PCR products of field samples and controls, primers IS*1296* and R1. Lanes: M = Molecular weight markers (MWM; Bio-Rad 60 – 2000 bp); 8 = *Mmm*; 15 = *Mmm*; N = negative control; and P = positive control (*Mmm*)

Identification and differentiation of Mmm

The genomic DNA of the two detected *Mmm* strains and controls were further specifically identified by using insertion element IS*1296* and R(all) primers pair. Positive amplified products of genomic DNA *Mmm* strains from the two field samples and positive control were identified as indicated by production of 1.1 kb fragment bands (Plate 2). However, PCR amplification using IS1296 element and R1 primer pair differentiated the two identified

Mmm strains genomic DNA and positive control to be of African cluster, as indicated by production of 1.4 kb fragments of agarose gel (Plate 3). Further, PCR amplification using IS*1296* element and R2 oligaonucleotides pair did not produce any amplicon.

Discussion

The geographical lineage of *Mmm* strains identified in pastoral cattle productions of Niger State, Nigeria was established. The first assay had allowed for specific detection of two *Mmm* antigens in the field samples and the positive control. The second assay differentiated them to be of African origin. The PCR tests have shown specific identification of these antigens at individual animal level as previously reported (Bashiruddin *et al*., 1994; Le Grand *et al*., 2004; Solano *et al*., 2005; Enyaru *et al.,* 2012). The molecular detection of *Mmm* strains in this circumstance was rapid and completed within 24 hours, making them very suitable tests for CBPP surveillance. This short outcome time observed was similar to the report of Bashiruddin *et al.* (1994), in which a similar PCR protocol with same primer pairs was completed in 48 hours with a specific detection of CBPP causative agent directly from tissues of suspected cattle. Culturing or enrichment was not carried out in the present survey. Culturing has not always been a successful epidemiological surveillance method, especially where animals have been treated with antibiotics, corticosteroids, or both (Bashiruddin *et al.,* 1994), which are common practices by animal health professionals in the state.

Identification and differentiation of *Mycoplasma myoides* subsp. *myoides* strains and geographical lineages have not been previously possible due to lack of appropriate markers and methods. Ribotyping, which is generally the method of choice for genetic subtyping of strains, is not applicable in this case because *Mycoplasma* species have a low number of *rrn* genes. However, Insertion Sequence (IS) fingerprinting has proved to be particularly useful for sufficient discrimination between strains and for lineages (van Embden *et al.,* 1993; Stanley *et al.,* 1993; Small *et al.,* 1994) using polymerase chains reactions (Miles *et al*., 2006).

The differentiation of these strains of African origin was achieved through oligonucleotide R1 binding within a deleted region in the bacteria, which is not present in strains of European strains. The lack of a binding site in European strains results in absence of PCR product in the third stage of fingerprinting protocol. Although primer R2 has a binding site in African strains, no fragment was generated since the primers are separated by over 10 kb segment. The short extension time and activity of the polymerase enzyme also excluded the production of European strains fragment. Absence of an 8.84 kb deleted region within European strains has, therefore, been identified as the cause of their variability from the African strains (Cheng *et al.,* 1995; Vilei *et al*., 2001; Vilei *et al.,* 2010). Localization of IS*1296* upstream of this deletion point has been considered sufficiently stable for species typing (Mahillon & Chandler, 1998;

Miles *et al.,* 2006) thereby precluding the use of more cumbersome methods of endonuclease analysis for species differentiation of *Mmm* (Bashiruddin *et al.,* 1994; Miserez *et al*., 1997; Vilei & Frey, 2010). Typing of genome DNA extracts over the deleted region using PCR assay considerably reduces the time and sample manipulation required for analysis, which have been previously reported to be critical challenges with restriction fragment length polymorphism (RFLP) and Southern blotting tests (Cheng *et al.,* 1995).

Previous research reports have also highlighted some bacterial strains, such as *Mycobacterium tuberculosis*, differentiation on the basis of PCR analysis using insertion-specific oligonucleotides (Suzuki *et al.,* 2004; Westberg *et al.,* 2004; Ablordey *et al.*, 2005). Further, geographical lineages of vaccine strains of *Mmm* analyzed with particular IS*1296* patterns have been reported (Cheng *et al.*, 1995). The differentiations of genomic DNA of *Mmm* strains from the field samples on the basis of presence or absence of the 8.84 kb deletion prove important for tracing origins of CBPP since the genes within this region are believed to be important in the virulence of this sub-species (Vilei & Frey, 2001).

The study has shown that the tests may be performed in concert on chronically infected animals for active surveillance of CBPP especially in endemic situations using field samples without enrichment or culturing. Non reliability on viable *Mmm* as in this situation, have been reported to be favorable (Bashiruddin *et al.*, 2005). Polymerase chain reactions have been reported to be the most successful detection method for *Mmm* due to high amplification factors of between 10^5 and 10^6 (Maes *et al.,* 1990; Bashiruddin *et al.,* 1999). This high fold sensitivity may have afforded possible specific identification of *Mmm* achieved.

The ability of a surveillance system to detect *Mmm* in cattle is a requirement of the World Organization for Animal Health for countries that wish to declare freedom from CBPP under the recommended standards for epidemiological surveillance systems for the disease (OIE, 1997). The use of PCR to detect *Mmm* strains and their geographical origins is the first report in Nigeria as no report of this nature, to our knowledge, has been documented in the recent past and this makes the present investigations unique to mitigate the disease in the country.

In conclusion, the fingerprinting PCR assay has provided for specific identification of *Mycoplasma mycoides* subsp. *mycoides* strains and, therefore has diagnostic power to detect these strains during active surveillance of the disease especially in sub-clinical and chronic stages. For routine active surveillance of the disease, these PCR tests are sufficient to detect the infections within a very short time with better outcomes than the traditional methods of culture and isolation, and identifications using biochemical and serological techniques. The presence of *Mycoplasma mycoides* subsp. *mycoides African*/Australian strains, which is useful for vaccine production, was therefore established in Niger State, Nigeria.

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