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*Full Length Research Paper*

# Macro-restriction analysis of *Staphylococcus aureus* isolated from subclinical bovine mastitis in Nigeria

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**Different pathogens cause both clinical and subclinical mastitis and *Staphylococcus aureus* is the most common. One hundred and thirty six apparently healthy cows from six Local Government Areas of Plateau state, Nigeria were sampled for the isolation of this pathogen. Three hundred and thirty nine quarter milk samples from the cows were collected, out of which 102 *S. aureus* were isolated. Twenty isolates were further analysed at molecular level. The species of the 20 strains were confirmed by PCR amplification using *S. aureus* specie-specific primers derived from the 23S rDNA and by amplification of *nuc*, *coa* and *spa* genes. Epidemiological relationships of the strains were studied by macro-restriction analysis of their chromosomal DNA using pulse field gel electrophoresis (PFGE). Among the 20 *S. aureus* strains identified, PFGE revealed an identical DNA pattern for 18 strains while two strains differed in two bands. These differences were revealed by the amplification of the *spa* gene. The relationship between the *S. aureus* isolated from nomadic raised cows discovered in the study areas remains unclear as only two pulse types were observed.**

**Key words:** *Staphylococcus aureus*, subclinical mastitis, pulsed-field gel electrophoresis, macro-restriction.

## INTRODUCTION

Mastitis is the most significant cause of economic loss in the dairy industry, and *Staphylococcus aureus* is known as an important causative agent all over the world (Cabral et al., 2004). *S. aureus* is known to cause both clinical and sub clinical mastitis. In Nigeria, Ameh et al. (1999) reported 34.6% *S. aureus* recovery from bovine mastitic milk in Maiduguri, while prevalence rate of 31% and 3.2% were reported by Umoh et al. (1990) from settled and nomadic herds respectively in Zaria, Nigeria. Phenotypic and genotypic characterisation of *S. aureus* isolates can be helpful in the development of more effective control practices for this disease. Some studies are consistent with the hypothesis that bovine mastitis is caused by few specialised strains that have a broad dissemination (Fitzgerald et al., 1997; Zadoks et al., 2000), whereas other studies have suggested that strains are more likely to be restricted to a single herd (Joo et al.,

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2001). However, all these studies have reported predominant strains responsible for bovine mastitis within herds (Matthews et al., 1994; Fitzgerald et al., 1997; Zadoks et al., 2000; Joo et al., 2001).

Data on phenotypic and genotypic characteristics of *S. aureus* isolates recovered from milk of cows with mastitis in Nigeria are still very limited (Umoh et al., 1990). Therefore, the purpose of the present study was to determine whether mastitis is caused by genetically related *S. aureus* strains in Nigeria as (observed in other countries). This knowledge will certainly be relevant in the development of mastitis control strategies directed against these specific strains.

## MATERIALS AND METHODS

### Distributions of milk samples

Bovine milk samples (n=339) were collected from 136 apparently healthy cows reared by nomads from six Local Government Areas in the Northern Senatorial Zone of Plateau state. These are; Jos East (n=18), Jos South (n=25), B-Ladi (n=20), Bassa (n=20), Riyom (n=23) and Jos-North (n=30). The locations were in a distance range of 10 to 30 km.

### Screening for sub-clinical mastitis

California mastitis test (CMT) was performed as a cow-side test evaluated as described by Umoh et al. (1990). Based on the thickness of the gel formed by CMT reagent and milk mixture, test result were scored as 0 (negative or trace), + (weak positive), 2+ (distinct positive), 3+ (strong positive).

### Sample collection

All quarter samples were collected according to the following procedure: prior to sampling the teats, udder and the adjacent flank areas were washed savlon disinfectant and dried with a single-service sanitary paper towel. About 15 ml of milk was collected into a sterile sample container directly from the udder and stored in an icebox and transferred to the laboratory within six hours.

### Bacterial isolation and identification

Microbiological culturing was carried out according to the standard protocols of Hogan et al. (1999). Sterile aluminium bacteriological loop was used to spread 300 µl of each milk sample onto blood agar (BA) (Oxoid), incubated at 37°C for 24h or 48h when growths were not observed. The suspected colonies on blood agar were further purified on Baird parker agar (Oxoid, Basingstoke, UK). Suspected colonies were gram stained and tested for catalase reaction. The hemolysis of the strains was determined on sheep blood agar. The staphylococcal strains were also investigated for hemolysis using the CAMP like test as described by Skalka et al., (1979). Coagulase and clumping factors were also investigated as previously described (Bruckler et al., 1994). Hyluronidase enzyme activity (Raus and Love, 1983) and acetoin production (VPR) with Methylrot Voges-Proskauer-Bouillon (Merck, Darmstadt, Germany) were also investigated.

In addition, twenty strains were investigated to confirm that the isolates were actually *S. aureus* using the commercial identification system Remel Staphaurex plus, (Remel, Kent, UK) and Staphytect plus (Oxoid, Basingstoke, UK), according to the manufacturers' instructions.

### Molecular identification and genotypic characterisation

Polymerase chain reaction (PCR) was performed on twenty isolates for the molecular identification and characterization.

### DNA extraction

DNA extraction was performed with DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions.

### Polymerase chain reaction

PCR was performed as described previously (Shuiep et al., 2009). The PCR reaction mixture contained 1.0 µl of each primer(10 pmol/ µl), 0.8 µl dNTP (10 mM; MBI Fermentas, St-Leon, Germany), 3.0 µl of 10x thermophilic buffer (Promega, Mannheim, Germany) with a final concentration of 1.8 µl $MgCl_2$ (Promega), 0.1ul Taq polymerase (5U/ µl; Promega/Boeringer) and 20 µl of water. Finally, 2.5 µl of DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling using (Gene Amp PCR System 2400, Perkin-Elmer, Rodgau Jugesheim, Germany). The presence of PCR products was determined by electrophoresis of 10 µl of reaction product in an 1.5% agarose gel (Gibco BRL, Karlsruhe, Germany) with Tris acetate electrophoresis buffer (TAE, 4.0 mmol/l Tris 1 mmol/l EDTA, pH 8.0) and gene ruler DNA Ladder Mix (Fermentas) as molecular size marker and visualized under UV (Image Master VDS, Pharmacia Biotech, Freiburg, Germany). Molecular identification by amplification of species-specific genes segment encoding staphylococcal thermo-stable nuclease (*nuc*) and coagulase (*coa*), the *spa* gene segment encoding the X region of staphylococcal protein A, were performed as had been described (Akineden et al., 2001; Cabral et al., 2004; El-Sayed et al., 2005; Shuiep et al., 2009). The primers and reaction conditions are as outlined in Table 1.

### Macro restriction analysis by pulsed field gel electrophoresis (PFGE)

The preparation of the DNA and the digestion with the restriction enzyme *Sma*I (Promega,) had been described previously (Cabral et al., 2004). The resulting fragments were subjected to pulsed field electrophoresis using the Chef Dr II system (Biorad, Munchen, German) with the following parameters: Voltage 6volts, an initial pulse of 5.3 s final pulse of 24.9s and 20 h migration at 14°C. The reaction patterns were analysed according to the recommendation of Tenover et al. (1995).

## RESULTS

From the 339 bovine mastitic milk samples obtained from 136 cows in the six Local Government Areas of Plateau State, Nigeria, 102 (30.3%) *S. aureus* were isolated. Twenty out of the 102 isolates were identified by their

**Table 1.** Primers and polymerase chaine reaction (PCR) programmes for amplication of genes encoding staphylococcal 23SrRNA and other genes.

| Gene | Primer | Primers Sequence | Amplicon sizeb (bp) | PCR programmes* | Reference |
|---|---|---|---|---|---|
| *23S rRNA* | *F<br>*R | ACGGAGTTACAAAAG GACGAC<br>AGC TCA GCC TTA ACG AGT AC | 1250 | 1 | Straub et al., 1999 |
| *Coa* | F<br>R | ATAGAGATGCTGGTACAGG<br>GCT TCC GAT TGT TCG ATGC | 500 | 2 | Hookey et al., 1998 |
| *Nuc* | F<br>R | GCGATTGATGGTGATACGGTT<br>AGCCAAGCCTTGACGAACTAAAGC | 280 | 3 | Brakstad et al., 1992 |
| *spa(IgG)* | F<br>R | CACCTGCTGCAAATGCTGCG<br>GGCTTGTTGTTGTCTTCCTC | 900 | 2 | Seki et al., 1998 |
| *spa(x- r)* | F<br>R | CAAGCACCAAAAGAGGAA<br>CACCAGGTTTAACGACAT | 300 | 6 | Frenay et al., 1996 |

*Prog nos. (1) 37 times (94°C, 40s; 64°C, 60s; 72°C, 75s); (2) 30 times (94°C, 60s; 58, 60s; 72°C, 60s); (3) 30 times (94°C, 120s; 55°C, 120s; 72°C, 60s); (6) 30 times (94°C, 120s; 55°C, 120; 72°C, 60s). *F- Forward primer. *R- Reverse primer.

hemolysis, positive coagulase, clumping factor, hyaluronidase, Dnase and Voges Preskauer reaction. In addition all the twenty isolates were confirmed to be *S. aureus* with the two commercial kits employed in this study. The species identity of all the 20 strains was confirmed by PCR- amplification of *S. aureus* specie-specific part of 23SrDNA and by amplification of the thermo nuclease encoding gene *nuc*. The amplification of staphylococcal coagulase encoding gene *coa* and the gene segment encoding the Xr-repetitive region of protein A encoding gene *spa* for all the 20 strains yielded uniform amplicon sizes of approximately 300bp. Amplification of the gene region encoding the lgG-binding region of protein A encoding gene *spa* for the 18 strains yielded amplicon size of 900bp and for two strains amplicon sizes of 750bp (Figure 1).

The clonal identity of all the 20 *S. aureus* from the present study was demonstrated by PFGE analysis. An identical DNA pattern was observed for the 18 *S. aureus* strains while DNA pattern of two strains differed in two bands (Figure 2).

## DISCUSSION

Clinical mastitis is often diagnosed in animals that show obvious signs of mastitis. These signs include swollen and painful udder which may be reddish and tender in appearance, while sub-clinical mastitis is diagnosed in apparently healthy animals which may not show any of the above mentioned signs. It is often possible that milk from one teat (quarter) out of the four teats in a cow could be mastitic and the others non mastitic. The 339 milk samples were collected from individual teats of the animals which explained the sample size and a CMT conducted. The CMT positive samples were subjected to isolation of *S. aureus* as previously reported (Umoh et al., 2009). The twenty out of one hundred and two isolates were randomly selected for further studies purely for ease of handling and economic reasons. The results of the PCR amplification of the nuc, coa and the spa genes agreed with previous researchers (Akineden et al., 2001; El-Sayed et al., 2005). PFGE was carried out to observe the epidemiological relationships of the strains, which is a gold standard for epidemiological studies of this nature, the differences in the pulse types in the two strain according to Tenover (1995) may be classified as closely related. This is because the number of pulse types observed was not enough to classify the two strains as significantly far related to the other eighteen isolates. The observation of two pulso types in this study is also in agreement with the observation of Fitzgerald et al. (1997) and Zadoks et al. (2000) who agreed with the hypothesis that bovine mastitis is caused by few specialised strains that have a broad dissemination, and also with other studies which suggested that strains are more likely to be restricted to a single herd (Joo et al., 2001). The report of eighteen types and two types agrees with studies which reported that predominant strains might be responsible for bovine mastitis within herds (Matthews et al., 1994; Fitzgerald et al., 1997; Zadoks et al., 2000; Joo et al., 2001). This also goes to confirm the isolation of 102 *S.*

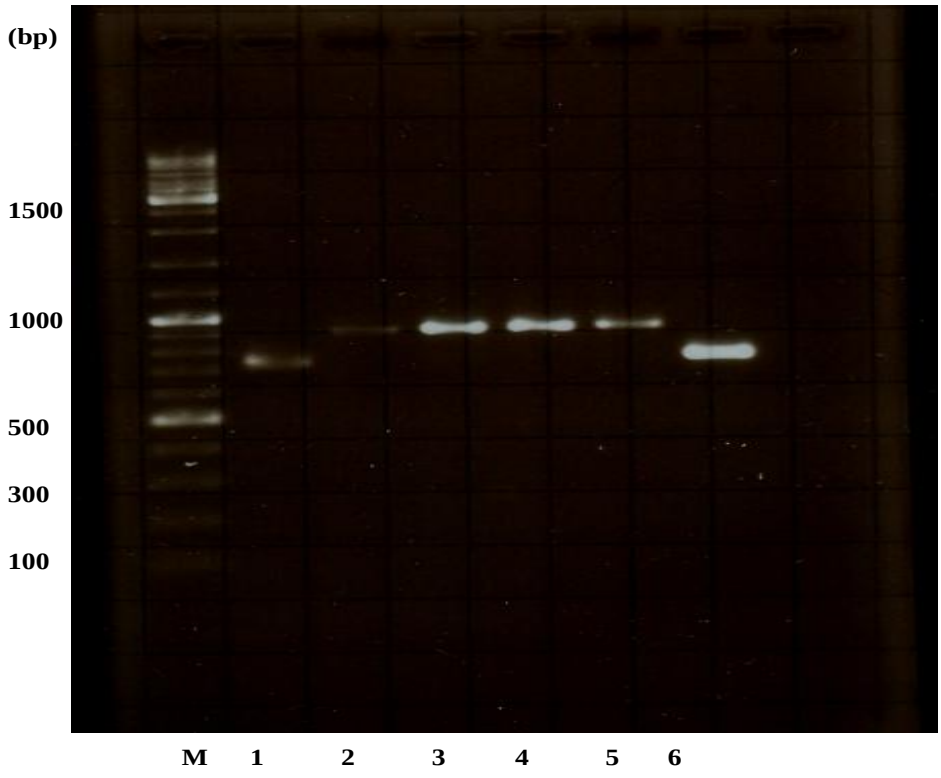


**Figure 1.** Amplicon of the gene encoding the I gG binding region of protein A. Lanes 1 and 6, 750 bp; lanes 2, 3, 4, and 5, 900 bp; M=a 100bp ladder served as size marker.

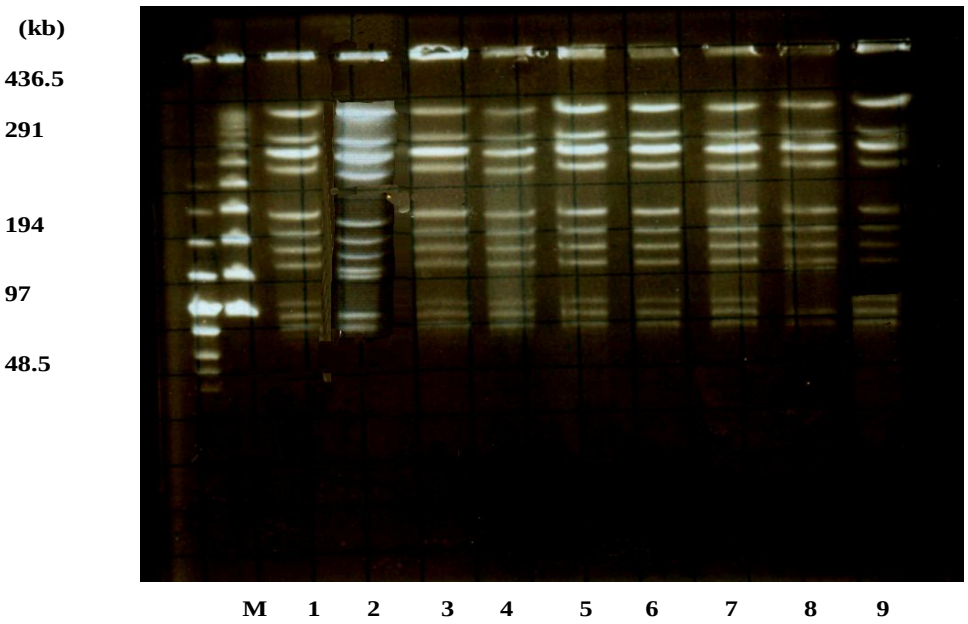


**Figure 2.** Pulsed-field electrophoretic restriction patterns of chromosomal DNAs of *S. aureus* isolates with DNA restriction patterns 1a (lanes 1, 3, 4, 5, 6, 7 and 9), 1b (lanes 2 and 8). M 0.1- to 200-kb ladder (Low Range PFG Marker; Biolabs, Schwalbach, Germany) and a 50- to 1000-kb ladder (Lamda Ladder PFG Marker, Biolabs) served as molecular marker.

*aureus* out of 105 mastitic milk samples.

The size difference observed by amplification of the gene segment encoding the l gG- binding region of protein A gene *spa* and the difference in DNA pattern of two strains compared to the remaining eighteen strains might be caused by evolutionary processes. The presence of an identical or closely related *S. aureus* clone for all isolates of the present study indicated that a direct transfer from animal to animal seems to be possible. However, the relationship of the nomadic raised cows originally found in six local government areas in Plateau state, Nigeria remains unclear. It may be concluded that a single clone is responsible for the subclinical mastitis in the area studied and therefore may required a single or similar treatment.

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