# Phenotypic Characterization of *Staphylococcus species* isolated from cases of bovine mastitis in parts of Plateau State, Nigeria

**Suleiman, A. B.,[1] Makolo, D..[2] Ahmad, A.E.,[3] Tahir, M. I.,[3] and Dikwa, K. B.[4]**

[1]Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria-Nigeria

[2]Department of Sciences, School of Preliminary Studies, Kogi State Polytechnic, Lokoja-Nigeria

[3]Department of Medical Laboratory Science, Faculty of Allied Health Science, Ahmadu Bello University, Zaria-Nigeria

[4]Department of Biological Sciences, Nigerian Defence Academy, Kaduna-Nigeria

**Abstract:** It has been established that bovine mastitis caused by *Staphylococcus aureus* is still a serious threat to dairy industry globally. Consumption of raw milk from the mastitic milk has been identified as a major source of public health issues in the developing nations. This study therefore was aimed at phenotypic characterization of *S. aureus* isolates from cases of bovine mastitis using both traditional, conventional and serological techniques. A total of 339 milk samples were collected from 98 cows at quarter level and analyzed for subclinical mastitis ~~by~~ California Mastitis Test (CMT). The CMT positive samples were bacteriologically analyzed following standard procedures for the isolation of *S. aureus*. Further identification and strain typing was done using Staphytect Slide Agglutination Test System and Microbact Staphylococcal 12S Identification System kits. Of the 339 samples analyzed, 30.9% were identified as subclinical mastitis. Moreover, 103(98.1%) of them harbored Staphylococcus species. Also out of the 40 randomly selected isolates that were strain typed, 39 (97.5%) were confirmed as *S. aureus* and 1 (2.5%) was *S. hyicus* and all the 40 isolates were coagulase positive. This study concludes that there is high prevalence of *S. aureus* in the mastitic milk samples studied and this poses a potential health threat not only to public health and safety of the consumers but also to the general public. It also identifies that that a reliable, rapid identification and strain typing of Staphylococcus species by both traditional, conventional and serological techniques provides a cornerstone for the control of *S. aureus* mastitis

**Key words:** *Mastitis, bovines, Staphylococcus aureus, Milk.*

## INTRODUCTION

Mastitis is a disease of economic concern in dairy herds involving the swelling of the mammary gland which often results in changes in the physical, chemical and bacteriological characteristics of milk amongst other symptoms (Radostis *et al*., 2000; Beheshti *et al*., 2010; Hussain *et al*., 2012). *Staphylococcus aureus* has been long implicated as a major agent of contagious bovine mastitis globally (Fox and Gay, 1993; Suleiman *et al*., 2012). Adhesion of *S. aureus* to mammary gland epithelium is considered to be a critical first step in the pathogenesis of mastitis (Cifrian *et al*., 1996).

Correspondence author:
sulybabs1@gmail.com

It is well documented that *S. aureus* produces several virulence factors, such as exoproteins and various cell surface proteins that contribute to the pathogenicity of this organism. Most of these virulence factors have been well characterized (Baba *et al*., 2002). Among the secreted staphylococcal virulence factors are the enterotoxins (SEA to SEE and SEG to SEQ), other toxins like exfoliative toxin A and B, toxic shock syndrome toxin, and the exotoxin-like protein family. *S. aureus* can also express several cell-wall-associated proteins with binding capacity for various host proteins, such as fibronectin, fibrinogen and collagen. In addition, *S. aureus* might express extracellular, antiphagocytic capsular polysaccharides (Baba *et al*., 2002).

Mastitis is a great problem among dairy herds globally as it adversely affects animal health, quality of milk, the economics of milk production and health of humans who consume the mastitic milk (Sharma, 2007).

There is a consensus of opinion among researchers of dairy herds that mastitis is the most wide spread infectious diseases in dairy cattle, and the most economically damaging (Tiwari *et al*., 2000; Halasa *et al*., 2007; Shittu *et al*., 2012; Suleiman *et al*., 2012)

Basically, the forms of the disease are the subclinical and clinical mastitis (Kivaria, 2006).Clinical cases of mastitis are characterized by the presence of one or more of the following: clots and/or blood in milk, udder swelling and systemic signs including an elevated temperature, lethargy and anorexia (Erskine, 2001; Suleiman *et al*, 2012). Sub-clinical cases show no visible changes in the appearance of the milk or the udder, but milk production decreases; composition is altered and is characterized by high somatic cell count (Erskine, 2001). The greatest risk of acquiring mastitis occurs in the first 50 days of lactation and in the early part of the dry period. The risk of clinical mastitis also increases with increasing parity (Erskine, 2001).

Somatic Cell Count (SCC) has been generally accepted as the best index to predict udder infection in bovines, and since the 1960s it has been extensively useful as an indicator of mastitis infections among the dairy herds (Pyorala, 2003; Kivaria, 2006). However, under research and field conditions, the use of California Mastitis Test (CMT) has found wide application due to its affordability, simplicity of usage, fast analysis of samples and usefulness in the selection of quarters for subsequent bacteriological examination (Kivaria, 2006). In spite of such success in the diagnosis of mastitis, significant success is yet to be achieved in the eradication and control of mastitis caused by *S. aureus* among dairy herds leading to relatively high culling rate and reduction in milk production (Sutra and Poutrel, 1990; Radostis *et al*., 2000; Suleiman *et al*., 2012). Thus, reliable, affordable and rapid methods of identification of *S. aureus* from cases of bovine mastitis remain crucial for effective control of the disease and economically sound udder health management (Boerlin *et al*., 2003). This study is aimed at characterizing *S. aureus* isolated from cows as a causative agent of bovine mastitis using the traditional, conventional and serological techniques .

## MATERIALS AND METHODS

### Study Area

The study was conducted in the Northern senatorial district of Plateau State, which lies between latitude $80^{0}24^{1}$ north and longitude $80^{0}$ $32^{1}$ east, of the equator. The area comprises of Jos South, Barkin Ladi, Jos North, Jos East, Riyom and Bassa Local Government Areas (Fig. 3.1). The area was selected because it has high population of cattle compared to other senatorial districts in the state.

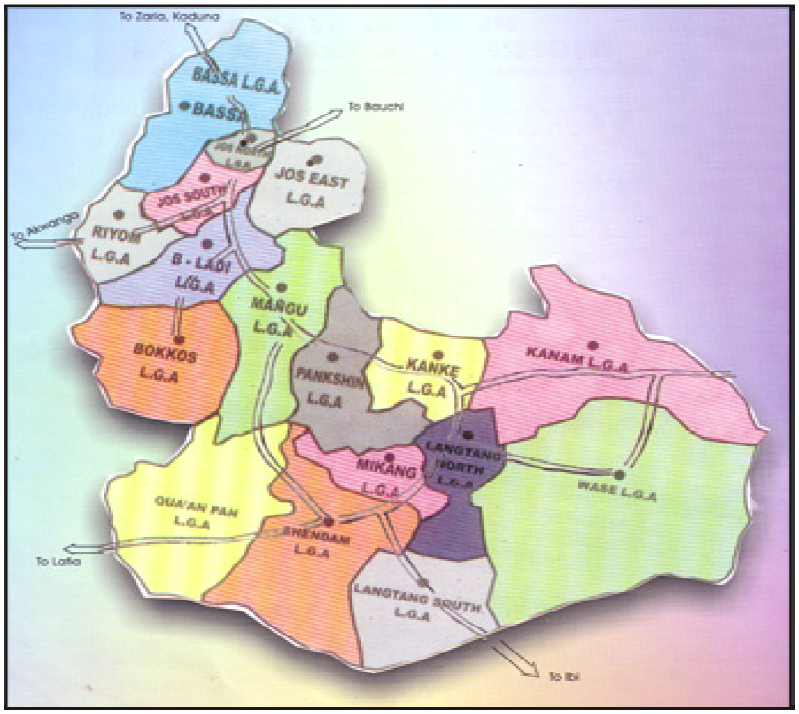


**Fig. 3.1: Map of Plateau State showing the study area indicated with red line**

**Study Design**

A cross-sectional study was carried out among lactating bovines from nomadic settlements within six Local Government Areas in Plateau State, Nigeria. The study animals were White Fulani cows, herded during the day time and in traditional enclosures overnight. The calves were separated from their dams during the night. A maximum of 30 lactating cows per herd were sampled randomly between April, 2018 and February, 2019.

**Sample Size Determination and Sampling Technique**

Two herds were selected from each local government area, from herd's owners who agreed to participate in the study from arrangement with the official contact and assistance of the extension services of National Veterinary Research Institute, (NVRI) Vom. A sample size of 339 quarter milk samples was used for this study based on a prevalence of 30% reported by Umoh *et al*. (1990a), in a study conducted in Zaria. The sample size was determined by this equation: $Z_{\alpha}{}^{2}{}_{(2).}PQ/L^{2}$ (Martin *et al*., 1987).

**Milk Sample Collection**

Prior to sampling, the udder, teats and adjacent flank areas were thoroughly washed and dried with single service sanitary paper towel. The teats were disinfected with 70% alcohol before sampling. About 15ml of milk samples were collected in sterile universal bottle directly from the udder after CMT reaction was carried out on all lactating quarters. Milk samples were transported in an ice box to the laboratory for microbiological analysis.

**California Mastitis Test**

Milk samples from each quarter were tested for sub-clinical mastitis using the CMT kit according to manufacturer's instructions on the field (Hogan *et al*., 1999; Quinn *et al*., 2002). The results for CMT reactions were recorded as 0 (negative), ±(trace), +1 (weak positive), +2 (distinct positive), +3 (strong positive). In this study CMT score of 0 or trace were excluded, because they were assessed as having originated from cows free of subclinical mastitis. While CMT result of ≥ + 1 were classified as evidence of subclinical mastitis as describe by Assefa *et al*. (2006) and Makolo *et al*., (2019).

**Isolation and Identification of *Staphylococcus aureus***

*Staphylococcus aureu*s was isolated from milk samples collected from dairy herds according to the protocols of the National Mastitis Council (Harmon *et al.,* 1990). An aliquot of 100µl from each sample was spread over blood agar plates (Bacto-Agar, Difco, Detroit, MI) containing 5% washed sheep erythrocytes and incubated at 37$^{o}$ C for 24hours. Colonies suspected to be staphylococci were sub-cultured on blood agar plates and isolation and identification of *S. aureus* was performed using the following test reactions: Gram staining reaction, coagulase test, growth on Baird-Parker medium, catalase test, Dnase test, Voges-Proskauer test, and mannitol fermentation test by standard bacteriological procedures. Typical isolates of *S. aureus* from the tests above were kept on nutrient agar slants for further study.

**Staphylococcus *aureus* Specific Latex Agglutination Test**

All the gram positive cocci that were catalase and coagulase positive were subjected to serological testing using the staphytect slide agglutination test system (Oxoid), which is a latex agglutination test that specifically identifies *S. aureus* from related bacteria. The procedure was conducted according to the Manufacturer's instructions (Oxoid).

**Confirmation of *S. aureus* using Microbact 12s Staphylococcal Identification System**

The *S. aureus* isolated were selected randomly and subjected to further characterization using Microbact Staphylococcal 12S Identification System Test, for the confirmation of the staphylococcal species. The test was conducted according to the instructions of the kit manufacturer (Oxoid Ltd., Basingstoke, and U.K).

## RESULTS AND DISCUSSION

The results of CMT conducted on 339 milk samples for the presence of sub-clinical mastitis are presented in Table 1. Considering CMT scores of negative (-) and plus/minus (±) as negative and 1+, 2+ and 3+ as positive, 105 (30.9%) quarter milk samples were found to be CMT positive and 234 (69%) were CMT negative. The prevalence of subclinical mastitis ranged from 25.7% to 38.0% between herd locations (Table 1). Overall, CMT results of ±, +1, +2, +3 were observed in 24%, 22%, 39% and 44% of quarter milk samples respectively. Also, 9.1% of the 22 milk samples with 1+ CMT positive score yielded no growth of *S. aureus,* while ~~20 (~~90%~~)~~ were *S. aureus* positive. Thirty nine (39) and 44 milk samples with 2+ and 3+ CMT positive respectively, were also *S. aureus* positive (Table 2).

**Table 1: California Mastitis Test Scores of Milk Samples Obtained From Different Local Government Areas From Northern Part of Plateau State**

| Location of herds (Local Govt.) | No. Tested | CMT Results/Scores | | | | | Σ(CMT≥+) (CMT) Positive) | Prevale (%) |
|---|---|---|---|---|---|---|---|---|
| | | - | ± | + | ++ | +++ | | |
| Jos South | 61 | 33 | 11 | 4* | 10* | 3* | 17 | 27.9% |
| Barkin Ladi | 61 | 39 | 4 | 5* | 10* | 3* | 18 | 29.5% |
| Bassa | 47 | 27 | 3 | 7* | 3* | 7* | 17 | 36.2% |
| Riyom | 66 | 42 | 2 | 3* | 6* | 13* | 22 | 33.3% |
| Jos East | 34 | 18 | 3 | 0 | 6* | 7* | 13 | 38.2% |
| Jos North | 70 | 51 | 1 | 3* | 4* | 11* | 18 | 25.7% |
| Total | 339 | 210 (61.9%) | 24(7%) | 22 (6.4%) | 39 (11.5%) | 44 (12.9%) | 105 (30.9%) | 30.9% |

Note: the relationship between CMT Score and SCC according to https://extension2.missouri.edu/g3653 is given below:

1) Negative (-) = <200,000 cells/ml (Healthy Quarter)
2) Trace (±) = 150,000 – 500,000 cells/ml (Subclinical Mastitis)
3) Weak Positive (+1) = 400,000 – 1,500,000 cells/ml (Subclinical Mastitis)
4) Distinct Positive (+2) = 800,000 – 5,000,000 cells/ml (Serious Mastitis Infection)
5) Strong Positive (+3) = Over 5,000,000 cells/ml (Serious Mastitis Infection)

**Table 2: CMT Scoring in Comparison with frequency of isolation of *S. aureus* from Cow Milk**

| | | Number of Samples (%) | |
|---|---|---|---|
| **CMT Score** | **Examined** | ***S. aureus* Positive** | ***S. aureus* Negative** |
| $1^+$ | 22 | 20 (90.9%) | 2(9.1%) |
| $2^+$ | 39 | 39 (100%) | 0 (0%) |
| $3^+$ | 44 | 44 (100%) | 0 (0%) |
| Total | 105 | 103 (98.1%) | 2 (1.9) |

Of the 105 mastitic quarter milk samples tested, 103 *S. aureus* were isolated, all were gram positive cocci, catalase positive, showed a typical growth on Baird Parker medium, were coagulase and clumping factor positive and all fermented mannitol. A variation of the haemolytic patterns of the isolates were observed; 40 (39%) of the isolates showed alpha haemolysis, 20 (19%) were beta haemolytic and 43 (42%) were gamma haemolytic. Eighty (78%) of the isolates demonstrated a DNase activity and 90 (87%) of the isolates were Voges-Proskauer positive. All the 103 Isolates were confirmed with the staphytect plus agglutination test system (Oxoid) as coagulase positive *S. aureus* (Table 3).

**Table 3: Main Biochemical Characteristics of 103 S. *aureus* isolates from Milk Samples tested**

| **Test** | **No. (%) positive** |
|---|---|
| Haemolysis | 103(100) |
| Alpha | 40 (39) |
| Beta | 20(19) |
| Gamma | 43 (42) |
| Coagulase | 103 (100) |
| Clumping factor | 103 (100) |
| DNase | 80 (78) |
| Voges-Proskauer | 90 (87) |

Fourty of the isolates previously identified as *S. aureus* according to conventional techniques (Harmon *et al.,* 1990), were subjected to Microbact test (Oxoid). Thirty nine isolates were confirmed as *S. aureus* and one isolate as *S. hyicus.* Six biochemical patterns were identified for the 39 *S. aureus* isolates, represented by the capital letter A to F (Table 4). All the isolates fermented maltose, mannose, sucrose and trehalose and produced arginine dihydrolase. Isolates varied in the following biochemical reactions: production of urease, β-glucuronidase, β-galactosidase and fermentation of mannitol and glucosamine. The biochemical pattern C was predominant, being observed in 25 isolates (64.1%) and distributed in all the herds studied. Two distinct patterns, D and F were found in each herd (Table 4).

**Table 4: Phenotypic Typing of 40 putative *S. aureus* Isolates from Bovine Mastitis using Microbact 12S***

| Patterns | No. of Isolates (%) | Maltose | Mannitol | Mannose | Sucrose | Trehalose | N-glucosamine | Argnine | Urease | β –Glucuronidase | β – Galastosidese | A- phosphatase | β Glucosidase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 2 (5.1) | + | + | + | + | + | + | + | + | + | ± | + | + |
| B | 8 (20.5) | + | + | + | + | + | + | + | + | - | ± | + | + |
| C | 25 (64.1) | + | + | + | + | + | + | + | - | - | - | + | + |
| D | 1 (2.6) | + | + | + | + | + | - | + | + | - | - | + | + |
| E | 2 (5.1) | + | + | + | + | + | + | + | + | - | - | + | + |
| F | 1 (2.5) | + | - | + | + | + | + | + | + | - | - | + | + |
| **Total** | **39(100)** | | | | | | | | | | | | |

## DISCUSSION

In this study, the prevalence of subclinical mastitis based on CMT, was found to be 30.9%. This is much higher than the prevalence of 3.2% reported among nomadic herds by Umoh *et al*. (1990a). Previous studies in small holder dairy cattle also reported a high prevalence of subclinical mastitis (Shekimweri, 1992; Omore *et al*, 1996; Karimuribo, 1999) as well as large dairy herds (Kinabo and Assey. 1982).

This study demonstrated an association between CMT scores and *S. aureus* intramammary infection (IMI). This agrees with other findings, which indicated that IMI is the most important variable affecting somatic cell count (Radostits *et al*., 2000). A threshold of 500,000 cells $m^1$ has been accepted as an indication of IMI (IDF, 1979; E.U, 1992). This limit was set, considering that SCC is indicative of herd mastitis control, hygienic milk production conditions and suitability of milk for use in manufacturing. However, there is no general agreement about what can be considered as a normal SCC for an infected quarter or cow (Harmon, 2001).

Likewise, there is a dearth of comprehensive studies that set criteria to define a normal SCC for low yielding cows, taking into account differences in breed, geographical area, husbandry and management conditions and type of milking.

The fact that, in the present study, 98% of the samples in which *S. aureus* was isolated had CMT scores ≥ +1, suggest CMT values as indicator for the presence of *S. aureus* IMI, in the dairy cows included in this study. Accordingly, it can be said that CMT score of +1 corresponding to 500,000 somatic cells $m/s^{-1}$ is less than one-tenth as likely to come from infected quarters as from non-infected quarters. Conversely, it can be predicted that SCC higher than 800,000 cells $m/^{-2}$ or a CMT score of ≥+2 is at least five times as likely to come from infected quarters as from uninfected quarters. These results further support the CMT as screening tool for subclinical mastitis in low-yielding quarters at herd level.

Furthermore, Low proportions of false positives were found in the present study. This suggests that CMT and microbiological screening are the most reliable indicators of on-going IMI.

The CMT-positive and culture-negative samples could be explained partly by the udder trauma or effect of antibiotic treatment or the infection could have been due to non-bacterial pathogens (Menziesand Ramanoon, 2001), or other bacterial pathogens other than *S. aureus*.

A reliable and rapid identification of *S. aureus* colonies in cultures from milk samples is a cornerstone in the control of *S. aureus* mastitis (Boerlin *et al*., 2003). The high specificity and sensitivity of the coagulase test (Blobe and Schlisser, 1994) has made it to be considered a standard method for the identification of *S. aureus.* In this study 100% of the *S. aureus* were positive in the coagulase test after 4-hour incubation. Beta haemolysis also represents an important criterion for rapid presumptive identification of *S. aureus* in primary cultures. The results of the present study showed a β-hemolytic activity in approximately one fourth of the *S. aureus* tested. This, however differs from previous studies which reported that one fifth of the *S. aureus* isolates from bovine mastitis do not present any detectable beta-haemolytic activity in primary cultures (Lam *et al*., 1995; Aarestrup *et al*., 1999).

Moreover, the combination of β-haemolytic test with coagulase test may provide a more sensitive and acceptably rapid identification method. The proportion of beta-haemolytic *S. aureus* found in bovine mastitis has been found to vary from region to region (Larsen *et al*., 2002). The performance of this combination in terms of sensitivity may also vary based on the *S. aureus* population under investigation and should therefore be interpreted with caution. Because of the frequent presence of a weak DNase activity in many species other than *S. aureus* (mainly in the alpha–haemolytic *S. haemolyticus*), the interpretation of DNase tests remains partially subjective and needs some experience. In this study, 78% of the isolates were DNase positive, and 87%. *S. aureus* were hyluronidase positive. In another study conducted elsewhere, 25 out of 28 *S. aureus* were found to be hyluronidase positive (Shuiep *et al.,* 2009).

Validation of the Staphytect slide test with conventional biochemical test for *Staphylococcus aureus* in this study showed a very satisfactory result with a concordance of 100%. The result of this study is not in agreement with the report of Boerlin *et al*. (2003), who reported a 62.7% for *S. aureus* from bovine mastitic milk. Other previous studies on *S. aureus* of human origin gave sensitivities and specificities of more than 98% (Persone *et al*., 1997; Gupta *et al*., 1998; Smole *et al.,* 1998). This could mean that the *S. aureus* isolated from this study may be of human origin.

The Microbact 12S kit method proved successful in identifying 39 out of 40 (97.5%) of the bovine *S. aureus* on which it was applied. One isolate was identified as *S. hyicus,* which was initially identified as *S. aureus* by conventional biochemical testing. This discrepancy can be explained by the fact that conventional tests failed to distinguish between *S. aureus, S. intermedius* and *S pseudintermedius* (Sasaki *et al,* 2007b). Colony morphology on blood agar, pigmentation and haemolysis used in routine diagnosis are not sufficient for species differentiation of coagulase positive staphylococci (CPS); (Hogan *et al.,* 1999; Bannerman and Peacock, 2007). Several commercial identification systems have been used to improve the identification of CPS with accuracy (Lammler, 1989; Watts and Yancey, 1994; Bascomb and Manafi, 1998). However, the differentiation accuracy varied markedly (38% - 91%) both when human and animal isolates were tested (Watts and Nickerson, 1986; Watts and Yancy, 1994; Bascomb and Manafi, 1998).

In addition, a diversity of biochemical patterns was observed among tested strains. In the present study two strains were urease negative and one each failed to ferment mannitol and utilise N-acetyl glucosamine respectively. This is similar with other reports, suggesting that some strains have atypical biochemical patterns (Lange *et al*., 1999; Tollersrud *et al*., 2000).

Understanding of atypical biochemical patterns is important so that isolates are not misidentified. Misidentification could lead to a decrease in the true number of isolates in a specific study. Therefore, isolates with atypical patterns should be only excluded after the use of other identification methods including molecular methods.

## CONLUSION

This study concludes that the prevalence of subclinical bovine mastitis by CMT was found to be 30.9%. The conventional biochemical characterization, Staphytect slide agglutination kit (Oxoid) and microbact 12S staphylococcal identification kit, are useful in the identification of mastitis mediated *S. aureus*. Overall, the study identifies that a reliable, rapid identification and strain typing of Staphylococcus species by both traditional, conventional and serological techniques provides a cornerstone for the control of *S. aureus* mastitis. Most, importantly, it emphasizes the relevance of phenotypic characterization of *Staphylococcus aureus* associated with bovine mastitis which could lead to the development of improved strategies for the control of mastitis.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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