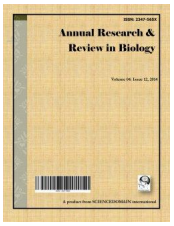

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Characterization of *Staphylococcus aureus* Small Colony Variant (SCV) Clinical Isolates in Zaria, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author ROI designed the study. Author BOO performed the statistical analysis. Authors ROI and JOE wrote the protocol and wrote the first draft of the manuscript. Authors ROI and BOO managed the analyses of the study. Author JOE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Staphylococcal isolates from specimen submitted to the Medical Microbiology laboratory of Ahmadu Bello University Teaching Hospital, Zaria were collected over a period of 6 months (February-July 2012), characterized by microbiological standard procedures and the *S. aureus* small colony variant (SCV) isolates were isolated. The antibiotic susceptibility pattern of the isolates was determined by the Kirby-Bauer-CLSI modified disc agar diffusion (DAD) technique. The SCV isolates were assessed for the carriage of four virulence genes; *sdrE* (putative adhesin) *icaA* (intracellular adhesin) *hlg* (hemolysin), *Cna* (collagen adhesin). A total of 258 non-duplicate staphylococcal isolates made up of 219 (84%) *S. aureus* and 39 (15%) coagulase-negative staphylococci (coNS) where obtained. A total of 48 (22%) isolates were determined to be *S. aureus* SCV mainly from

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wound/abscess (31%). *S. aureus* SCV isolates were generally resistant to all the nine antibiotics tested with only minimal sensitivity to tigecyclin (10.4%) and ciprofloxacin (18.8%). Statistically, there was no significant difference between the microbial load and the different antibiotics that were used, (P ≥0.05). None of the *S. aureus* SCV isolates carried the four virulence genes which were tested in this study. The results have therefore proved that *S. aureus* small colony variant exist in our environment and they are more resistant to most antimicrobial agent than their wild type.

Keywords: Staphylococcal; small colony variant; susceptibility; intracellular adhesion; collagen adhesion; hemolysin; putative adhesion; sensitivity.

1. INTRODUCTION

Staphylococcus aureus small colony variants (SCVs) are broadly defined as slow growing colonies with diameters roughly one tenth the parental strains when cultivated on agar plates [1]. Of particular importance are their decreased susceptibility to antibiotics and the absence of routine testing in clinical samples to detect their presence. These characteristics of SCV coupled with their capacity to revert to more rapidly growing form render them ideal candidates to provoke persistent human infections. Several decades of research now attest to their likely involvement in disease pathology [2,3].

Staphylococcus aureus SCVs are generally reported to be auxotrophic for compounds that are biosynthesized into components of the electron transport system [4,5]. Menadione and hemin are the two most frequent substances that reverse the *S. aureus* SCV phenotype [6,7]. Reduced activity of the electron transport system can account for most of the features of the *S. aureus* SCVs. For example, a reduction in available ATP would slow growth, reduce pigment formation and decrease aminoglycoside transport.

Persistence and therapy refractory courses are characteristic features of *S. aureus* SCV infections which represent a serious difficulty in treating clinical cases [1,8]. In general, *S. aureus* SCV diseases show a wide variety of manifestations, ranging from superficial skin infection to life threatening conditions such as septicemia [9,10]. In particular, endovascular diseases such as endocarditis are frequently caused by *S. aureus* and in many clinical institutions *S. aureus* SCV has evolved as the leading pathogen of these infections [2].

S. aureus chronic and therapy refractory infections, as well as intracellular persistence have been associated with the SCV phenotypes [1]. However, because clinical SCVs are difficult to detect and are usually not stable but rapidly revert to their originally wild phenotype, the host cell response to SCVs is largely unknown [2]. When located intracellularly, SCVs has been reported to avoid activation of the host innate defense system and do not kill the host cells during persistence. This can be explained by the down regulation of important virulence factors in SCVs (e.g. α- toxin and proteases), which normally contribute to inflammation and tissue destruction [11].

In chronic infections, *S. aureus* SCV persists mainly intracellularly, where the bacteria are well protected against most antimicrobial treatments and against the host innate defense system [12]. There is even some preliminary evidence that the endothelial intracellular environment may favour the development of SCVs and bacterial regulatory processes due to non-protein coding RNAs and this might play a role in the formation of SCVs [9]. The intracellular SCVs contribute significantly to pathology and their reduced antibiotic susceptibility heralds a serious clinical problem.

S. aureus remain very versatile and exist almost everywhere including the hospital settings; therefore, this work aims at characterizing *S. aureus* small colony variant clinical isolates from Ahmadu Bello University Teaching Hospital, Zaria.

2. MATERIALS AND METHODS

2.1 Culture Media

Mannitol Salt Agar (MSA); Nutrient Agar (NA); Nutrient Broth (NB); Mueller-Hinton Agar; Blood Agar Base; all from Oxoid, UK.

2.2 Antibiotic Discs

The following antibiotic discs from Oxoid, UK were used; Gentamicin [10 µg], ciprofloxacin [5 µg], vancomycin [30 µg] cefoxitin [30 µg], erythromycin [15 µg] clindamycin [2 µg],

tigecycline [15 µg], cefuroxime [30 µg], amoxicillin [30 µg] representing the members of penicillin, third-generation cephalosporin, aminoglycoside, fluoroquinolone and glycopeptide classes.

2.3 Collection of Clinical Isolates

Suspected staphylococcal isolate from specimens submitted to the Medical Microbiology laboratory of ABUTH, Zaria were collected on NA slants over a period of 6 months. The slants were incubated for 18 hours at 37°C until there was visible growth. Slants were kept refrigerated until needed.

2.4 Purification

All cultures on NA slants were subcultured into nutrient broth, incubated overnight and the resulting cultures were streaked on nutrient agar plates and purified by single colony isolation.

2.5 Preliminary Identification

A loopful of overnight NB culture of the isolates was streaked on previously prepared Mannitol Salt Agar (MSA) plates. The plates were incubated at 37°C for 24 h under aerobic condition. After 24 h of incubation, the culture plates were examined recording the appearance, size, colour, and morphology of the colonies. Gram stain reaction, catalase test and coagulase test were carried out. Isolates that were gram-positive cocci, catalase positive, and coagulated human plasma were considered *S. aureus* in this study.

2.6 Isolation of Small Colony Variants

2.6.1 Growth on blood agar

This was performed according to the method described by [12] Neut et al. (2003). A loopful of overnight nutrient broth cultures of confirmed *S.aureus* isolates were inoculated on a freshly prepared blood agar supplemented with 5% NaCl. The cultured blood agar plates were incubated in an inverted position. The incubation lasted for 48-72 hours at 37°C. Isolates that yielded non pigmented and non-haemolytic pin-point colonies were suspected to be small colony variants.

2.6.2 Auxotrophy assay

Auxotrophy was assayed by complementation with menadione sodium bisulphite (from Sigma-Aldrich AB, Stockholm, Sweden). This was performed as a confirmatory test for SCVs using a five millimetre diameter filter paper discs (3MM paper Whatman International Maidstone, United Kingdom) which were soaked in menadione bisulphite solution at a concentration of 200 µg/ml and aseptically placed with a forcep onto Mueller-Hinton plates inoculated with suspected *S. aureus* SCVs isolates.

Plates were incubated in inverted position aerobically for 24 hrs at 37°C. An increase in colony size proximal to the cellulose disc was interpreted as a positive result. This method was described by [6].

2.7 Antibiotic Susceptibility Testing

The antibiotic susceptibility pattern of the isolates was determined by the Kirby-Bauer-Clinical Laboratory Standards Institute (CLSI)-modified disc agar diffusion (DAD) technique. Discrete colonies of isolates on NA plate were emulsified in 3 ml of PBS and the turbidity adjusted to 0.5 McFarland. Using sterile swab sticks, the surface of MHA was inoculated with the bacterial suspension; the antibiotic discs were aseptically applied to the surface of the inoculated agar plates. Within 30 minutes of applying the discs, the plates were inverted and incubated aerobically at 37°C for 16-18 hours.

The diameter of the zones of growth inhibition were measured to the nearest millimeter and isolates classified as sensitive, intermediate or resistant based on CLSI interpretative chart of zone sizes [13].

2.8 Molecular Identification of Virulence Genes

2.8.1 DNA isolation and purification

The isolation and purification of genomic DNA from the isolates was done following miniprep method of [14] with modification.

2.8.2 PCR amplification of virulence genes

PCR amplification of four virulence genes was done as described by Peacock et al. [15]. Specific primer genes were used to amplify the genes. A 25 µl of reaction mixture was made containing 20 µg of template DNA, 100 µg of primers, 160 Mm of dNTP mix, 1.25U Taq polymerase, 1x Taq buffer and 0.5 Mm $MgCl_2$. All the *S. aureus* SCV isolates were amplified

Table 1. List of virulence genes and the primer sequences

Gene	Primer sequence	Amplicon size(bp)
cna (collagen adhesin)	F: 5'TTCGTCACAATCAAGTTTGCC3' R: 3'CGGTGAAAAAGTATGGGACG5'	744
hlg (hemolysin)	F:5'GCCAATCCGTTATTAGAAAATGC3' R: 3'CCATAGACGTAGCAACGGAT5'	937
icaA(intracellular adhesin)	F: 5'GATTATGTAATGTGCTTGGA3' R: 3'ACTACTGCTGCGTTAATAAT5'	770
SdrE(putative adhesin)	F: 5'AGTAAAATGTGTCAAAAGA3' R: 3'TTGACTACCAGGCTATATC5'	767

individually for four genes using the specific primers with 32 cycles of denaturation at 95°C for 1 min, annealing at 50°C for icaA, 45°C for sdrE and 55°C for hlg and cna for 1 min, extension at 72°C for 2 min on a thermocycler (PTC-100, MJ Research USA).

PCR products were resolved on 1.0% agarose gel at 60 volts for 2 hours. Gels were stained with ethidium bromide solution (0.5 µg/ ml) and documentation was done using the Gel Doc system (Bio-Rad).

2.9 Statistical Analysis

One-way ANOVA was used to analyze results of the microbial load of the SCVs staphylococcus aureus and the different antibiotics used based on Tukey-Kramer's Multiple Comparisons Test using InStat3 Software, 2013 (significant at $P<0.05$).

3. RESULTS

A total of 258 staphylococcal isolates were obtained from clinical specimen submitted to the Medical Microbiology Laboratory of ABUTH, Zaria over the period of 6 months. A total of 48/219 (22%) were determined to be *S.aureus* Small Colony Variants (SCV) phenotype. The distribution of SCVs by source shows that most of the isolates were from wound/abscess (31%) as shown on Fig. 1.

From the antibiotic Susceptibility test, the zone of growth of inhibition obtained was classified based on the CLSI Interpretative chart of Antimicrobial Sensitivity Testing. Table 2 shows the outcome. Table 3 shows the antibiotic susceptibility pattern of the *S.aureus* wild type. Compared to the SCV, the wild type *S. aureus* was more susceptible to ciprofloxacin and gentamicin antimicrobial agents. The prevalent resistant phenotypes for both the wild type *S.aureus* and the *S.aureus* SCV isolates where determined. Tables 4 and 5 show the outcome respectively. Fig. 2 shows the percentage resistance of *S. aureus* wild type and *S. aureus* SCV.

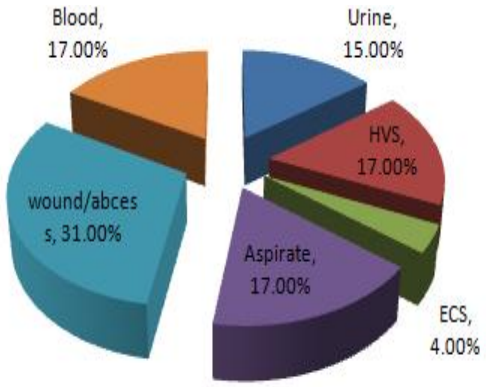

Fig. 1. Distribution of S.aureus SCV by specimen

4. DISCUSSION

The results from this work reveals that *S. aureus* small colony variant exist in our environment. The recovery rate of *S. aureus* small colony variants (SCVs) was 22% (48/219). The recovery rate in this study is in contrast to the report of [16]. He estimated the recovery rate of *S. aureus* SCVs in a general microbiology laboratory to be around 1%. Another study by Becker et al. [17] reported the recovery rate of *S. aureus* SCVs to be 14 isolates in a period of 3 years.

Analysis of the distribution of the *S. aureus* SCVs by source showed that majority were from wound/abscess (31%), blood (17%), HVS (17%), aspirate (17%) and urine (5%). The study of Gonzalez-Zorn and Courvalin [18] reported 5% and 3% recovery rate from blood and wound respectively.

Table 2. Susceptibility pattern of *S. aureus* Small Colony Variants isolates

Antibiotics	Disc potency	Resistant (%)	Intermediate (%)	Sensitive (%)
Tigecycline	15µg	79.2	10.4	10.4
Erythromycin	15µg	85.4	12.5	2.1
Amoxicillin	10µg	100	0	0
Cefuroxime	30µg	97.8	2.1	0
Gentamicin	10µg	83.3	16.7	0
Clindamycin	2µg	93.7	6.3	0
Ciprofloxacin	5µg	81.3	0	18.8
Cefoxitin	30µg	70.8	27.0	2.1
Vancomycin	30µg	-	-	6.3

Table 3. Antibiotic susceptibility pattern of wild type *Staphylococcus aureus* isolates

Antibiotics	Disc potency	Resistant (%)	Intermediate (%)	Sensitive (%)
Tigecycline	15µg	19.3	12.3	**68.4**
Erythromycin	15µg	55.6	19.9	24.6
Amoxicillin	10µg	49.1	14.0	36.8
Cefuroxime	30µg	72.5	15.8	11.7
Gentamicin	10µg	17.5	8.2	74.3
Clindamycin	2µg	59.1	9.4	31.6
Ciprofloxacin	5µg	20.5	11.7	67.8
Cefoxitin	30µg	44.5	26.4	29.2
Vancomycin	30µg	-	-	33.3

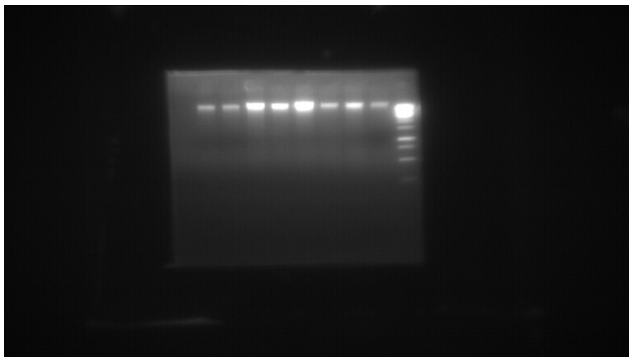

Fig. 2. Amplicon of the electrophoresis gel of the SCV *Staphylococcus aureus*

Table 4. Resistant phenotypes for wild type *S. aureus* isolates

Resistant phenotype	Number of isolates (n=171)	Percentage of isolates (%)
E, AML, CXM, CN, CIP, VA,FOX, DA	4	2
AML, CXM, CN, DA, FOX, E, CIP	4	2
E, AML,CXM, CN, CIP, FOX	18	11
CIP, VA, DA, CXM, AML	28	16
CXM, AML, FOX, VA	50	29
TCG, CN, DA	45	26
DA, E	11	6
AML	11	6

Key: TCG - Tigecycline, AML- Amoxicillin, DA - Clindamycin, E - Erythromycin, CXM - Cefuroxime, VA - Vancomycin, CN - Gentamicin, FOX - Cefoxitin, CIP - Ciprofloxacin

Table 5. Resistant phenotype of *S. aureus* SCV isolates

Resistant phenotype	Number of isolates (n=48)	Percentage of isolates (%)
TCG, AML,E, CN, DA, FOX, VA CIP, CXM	12	25
E, TCG, AML, CXM, CIP, CN,DA,FOX	18	38
AML, E, CN, DA, CIP, TCG, CXM,	10	20
TCG, E, AML, CXM, DA, CIP	6	13
AML, CXM, CN, DA, E	2	4

Key: TCG - Tigecycline, AML- Amoxicillin, E- Erythromycin, CXM - Cefuroxime, CN - Gentamicin, DA - Clindamycin, VA - Vancomycin, CIP - Ciprofloxacin, FOX - Cefoxitin

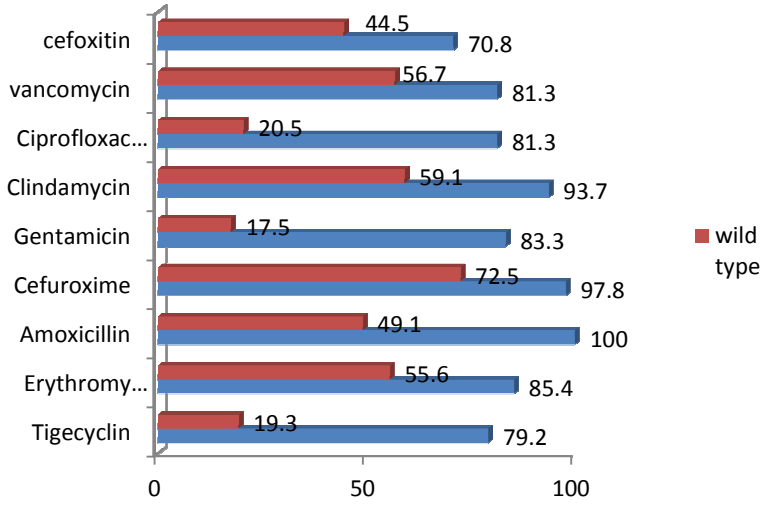

Fig. 3. Percentage resistance of *S. aureus* wild type and *S. aureus* SCV

Susceptibility testing of the small colony variant *S. aureus* isolates in this study against commonly available antibiotics showed that the isolates were generally resistant to β- lactam drugs; (amoxicillin, cefuroxime), gentamicin, erythromycin and vancomycin with minimal sensitivity to tigecycline and ciprofloxacin antibacterial agent. The high level of resistance of the *S. aureus* small colony variants to most of these commonly available antibiotics used in this study is in agreement with the report of Proctor and Peters [19] who concluded that the depressed electron transport activity seen in auxotrophic SCVs may account for their *in vitro* resistance to a variety of antibiotics. In addition, the low content of ATP in SCVs causes inefficient transport of aminoglycoside into the cell, resulting in increased resistance to gentamicin and other aminoglycosides [19]. Moreover, the slow growth of SCVs and consequently cell wall division, reduces the effectiveness of antibiotics that act at the cell wall [20].

The susceptibility testing of the wild type *S. aureus* isolates in this study against the same antibiotics showed that the isolates were generally resistant to β- lactam antibiotics (amoxicillin, cefuroxime), clindamycin, erythromycin and vancomycin while being generally sensitive to gentamicin (an aminoglycoside) and ciprofloxacin (a fluoroquinolone) antibacterial agents. In contrast to the result obtained in this study, [21] concluded that fluoroquinolones (e.g moxifloxacin) appeared consistently highly effective against the SCVs. Another study by Vaudaux et al. [22] reported that sensitivity to ciprofloxacin was higher for SCVs than for wild type *S. aureus* isolates with normal phenotype, while no remarkable difference was observed for other fluoroquinolones (moxifloxacin, levofloxacin and finafloxacin).

The susceptibility level of the wild type *S. aureus* to ciprofloxacin is lower than the 99.7% reported by Akerele et al. [23]. This development may be connected with the increasing availability of the cheaper generics of fluoroquinolones in this environment leading to mis-use, over-use and gradual development of resistance.

From the determination of the virulence genes present in the *S.aureus* small colony variant isolates it was observed that none of the four virulent genes which were tested was present in the small colony variant isolates. This finding is in contrast to that reported previously by [24] who isolated SCVs that were thymidine auxotrophs and showed the over expression of intracellular adhesin. Further work is thus needed to determine how intracellular adhesin is activated in some types of clinical SCVs and not others. One possible explanation for the lack of detection of intracellular adhesin in the SCVs may be the kinetics of gene expression over time [25].

5. CONCLUSION

Clinical and laboratory findings lead to the conclusions that SCVs must be actively sought after in clinical microbiology, because they grow very slowly and can easily be missed. Particularly samples from individuals suffering from unusually persistent or recurrent infections should be examined meticulously for SCVs. In addition, it is most important to take SCVs into account as a possible cause of persistent infectious diseases when no bacteria or unusual microorganisms are found from such clinical specimen. Also due to reduced production of virulence factors by SCVs, they are adapted to the intracellular environment for long term persistence. An optimal treatment of SCV mediated infections has not been established but the *S. aureus* SCV in this study shows increased resistance to aminoglycosides and cell wall active antibiotics. Thus further study can be done in this field of study in order to understand the factors which select these phenotypes in the host and the genetic basis of this type of auxotrophy (menadione auxotrophy).

ETHICAL APPROVAL

As per university standard guideline participant ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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