

Description of Methicillin-Susceptible *Staphylococcus aureus* Clonal Complex 30 Related to the Pandemic Phage Type 80/81 Isolated from Patients in Three Tertiary Hospitals in Jos, North Central Nigeria

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Highlights of the Study

- Twenty-two clonal complex 30-methicillin-susceptible *Staphylococcus aureus* isolates were investigated using multilocus sequence typing, *Staphylococcus* protein A typing, and DNA microarray analysis.
- The isolates belonged to sequence type 30 and five *spa* types: t012, t019, t017, t037, and t318 and were positive for enterotoxin genes (*seg*, *sei*, *seu*, *seo*, *sem*, *sen*).
- Eight isolates (36.3%) were positive for genes that code for Panton-valentine leukocidin.

Keywords

Methicillin-sensitive *Staphylococcus aureus* · Sequence type 30 · *spa* typing · Panton-valentine leukocidin · DNA microarray

Abstract

Objective: The prevalence of phage 80/81 *Staphylococcus aureus* strains, the pandemic strains that were dominant in the 1950s, had declined in the 1960s and 1970s. However, these strains have reemerged in some countries in recent years. This study investigated the antibacterial resistance, virulence, and the genetic backgrounds of CC30-MSSA isolates obtained from patients in three tertiary hospitals. **Materials and Methods:** Twenty-two CC30-MSSA isolates cultured from different clinical samples were investigated using

antibiotic sensitivity testing, *spa* typing, multilocus sequence typing, and DNA microarray analysis. **Results:** All 22 isolates were susceptible to vancomycin (MIC ≤ 2 $\mu\text{g}/\text{mL}$), teicoplanin (MIC ≤ 2 $\mu\text{g}/\text{mL}$), and cefoxitin but were resistant to penicillin G ($n = 22$; 100.0%), tetracycline ($n = 12$; 54.5%), ciprofloxacin ($n = 15$; 68.2%), cadmium acetate ($n = 22$; 100%), mercuric chloride ($n = 13$; 59.1%), and ethidium bromide ($n = 3$; 13.6%). The isolates belonged to sequence type, ST30, and five *spa* types: t012 ($n = 12$; 54.5%), t019 ($n = 5$; 22.7%), t017 ($n = 2$; 9.1%), t037 ($n = 2$; 9.1%), and t318 ($n = 1$; 4.5%). All 22 isolates were positive for *agrIII*, *cap8*, *clfA*, *clfB*, *icaA*, *icaC*, *icaD*, *cna*, and staphylococcal enterotoxin gene clusters (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*). Eight isolates carried *lukS-PV* and *lukF-PV* that code for Panton-Valentine leukocidin. **Conclusion:** The current CC30-MSSA isolates share phenotypic and genotypic characteristics with the pandemic phage 80/81 isolates that

were common in the 1950s and 1960s. Continued surveillance is recommended to keep abreast of the changing epidemiology of *S. aureus* causing healthcare and community-associated infections.

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Introduction

Staphylococcus aureus remains a leading cause of human infections worldwide. *S. aureus* can cause skin and soft tissue infections (boils, carbuncles, and pustules), pneumonia, and infections of the bloodstream, bone and joint, and respiratory and gastrointestinal tracts [1]. Several phenotypic and genotypic typing methods have been applied to characterize *S. aureus* isolates for their virulence properties, genetic relatedness, and antibiotic resistance determinants. These methods include phage typing, pulsed-field gel electrophoresis, staphylococcal protein A typing (*spa* typing), and multilocus sequence typing (MLST) [2, 3].

Bacteriophage typing (phage typing) was the primary method for typing *S. aureus* for epidemiological purposes in the 1950s–1970s [4, 5]. Phage typing is based on the ability of a set of bacteriophages (typing phages) to lyse *S. aureus* strains and produce patterns known as bacteriophage types [4, 5]. Bacteriophage typing was used to identify strains of *S. aureus* designated as phage 80/81 based on their susceptibility to lysis by a set of specific bacteriophages known as phages 80, 81, and sometimes phage 83 [2, 4, 5].

The phage-type 80/81 strains that were isolated in the 1950s were resistant to penicillin by producing penicillinase [2, 4, 5]. Some of the strains also produced a leukocidin designated Panton-Valentine leukocidin (PVL), a pore-forming cytotoxin that causes destruction of leukocytes and tissue necrosis [6]. In *S. aureus* strains, PVL is encoded by two co-transcribed genes, *lukS-PV* and *lukF-PV*, which are carried by bacteriophages [6]. The phage 80/81 strains were responsible for outbreaks of infections including skin and soft tissue infections (boils, carbuncles, and pustules), sepsis, and pneumonia in Australia, Great Britain, Canada, and the USA in the 1950s [2, 4, 5].

The emergence of penicillin resistance in *S. aureus* including the phage 80/81 strains led to the development and clinical use of methicillin, a penicillinase-resistant penicillin, to treat infections caused by penicillin-resistant phage 80/81 strains [1, 2]. The clinical use of methicillin subsequently resulted in the emergence of methicillin-resistant strains (MRSA), which has now become a

global health problem [1]. The emergence of MRSA and their global expansion coincided with the decline in the prevalence of the phage 80/81 strains [2] and the replacement of phage typing as an epidemiological typing tool by molecular typing methods such as *spa* typing and MLST [2, 3]. The application of MLST on the phage 80/81 isolates obtained in the 1950s and early 1960s revealed that they belonged to *S. aureus* clonal complex 30 (CC30) and sequence type sequence type 30 (ST30) [2, 7]. It was subsequently revealed that contemporary *S. aureus* CC30 isolates include the community-associated MRSA lineage ST30-MRSA-IV, also known as the Southwest Pacific clone, and the healthcare-associated lineage ST36-MRSA-II, which is also known as the UK Epidemic MRSA-16 [2, 7]. Whereas the CC30-MRSA strains have spread globally in recent years [8], the pandemic phage-type 80/81 strains have been reported sporadically in the literature [2, 9–11].

While investigating the genetic backgrounds of *S. aureus* isolates obtained from clinical samples in tertiary hospitals in Jos, Plateau State of Nigeria using DNA microarray, we detected methicillin-sensitive *S. aureus* (MSSA) strains that belonged to the CC30 lineage (CC30-MSSA). Here we report further investigations conducted on the 22 CC30-MSSA isolates using a combination of molecular typing methods to establish their genetic relatedness to the pandemic phage 80/81 strains.

Materials and Methods

Sources and Identification of Bacterial Isolates

The *S. aureus* isolates were collected as part of routine diagnostic microbiology service. Strains were identified based on growth characteristics on blood agar plate (5% sheep blood), positive Gram's stain, catalase, and tube coagulase tests. In total, 214 *S. aureus* were isolated in 2017 and 2018 in the Jos University Teaching Hospital (JUTH; $n = 8$), Plateau State Specialist Hospital (PSSH; $n = 12$), and Bingham University Teaching Hospital (BUTH; $n = 2$). The isolates were cultured from wound swabs ($n = 10$), blood cultures ($n = 9$), ear swab ($n = 1$), urethral swab ($n = 1$), and urine specimen ($n = 1$) obtained from 13 male and 9 female patients and were preserved in semisolid agar medium (0.3% agar in brain heart infusion broth). Molecular characterization was performed at the Gram-Positive Bacteria Research Laboratory, Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait.

Susceptibility to Antibacterial Agents

The disk diffusion and minimum inhibitory concentration (MIC) methods were performed according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [12]. The following antimicrobial disks (Oxoid) were tested: benzyl penicillin (10 U), cefoxitin (30 µg), kanamycin (30 µg), mupirocin (200 µg and 5 µg), gentamicin (10 µg), erythromycin (15 µg), clindamycin (2 µg),

Table 1. Antibiotic resistance determinants of CC30-MSSA isolates

Resistance profile	MSSA genotypes					
	CC30/ST30-MSSA-t012 (N = 12)	CC30/ST30-MSSA-t017 (N = 2)	CC30/ST30-MSSA, (PVL+)-t019 (N = 5)	CC30/ST30-MSSA, (PVL+)-t037 (N = 2)	CC30/ST30-MSSA, (PVL+)-t318 (N = 1)	N
Resistance phenotype						
P	12	2	5	2	1	22
TE	11	2	–	–	–	13
CIP	10	2	–	2	–	14
Cd	12	2	5	2	1	22
Hg	11	2	–	–	–	13
Eb	–	1	–	2	–	3
Resistance genotypes						
<i>blaZ</i>	12	2	5	2	1	22
<i>tet(M)</i>	11	2	–	–	–	13
<i>merA</i>	11	2	–	–	–	13
<i>merB</i>	11	2	–	–	–	13

merA, *merB*, mercury resistance gene; *blaZ*, beta-lactamase gene; *tet(M)*, tetracycline resistance gene; P, penicillin; TE, tetracycline; CIP, ciprofloxacin; Cd, cadmium acetate; Hg, mercuric chloride; Eb, ethidium bromide.

chloramphenicol (30 µg), tetracycline (10 µg), trimethoprim (2.5 µg), fusidic acid (10 µg), rifampicin (5 µg), and ciprofloxacin (5 µg). The MIC for oxacillin, ceftiofloxacin, mupirocin, vancomycin, and teicoplanin was determined with E-test strips (BioMerieux, Marcy l'Etoile, France). Susceptibility to fusidic acid was interpreted according to the British Society for Antimicrobial Chemotherapy [13]. *S. aureus* strains ATCC25923 and ATCC29213 were used as quality control strains for disc diffusion and MIC determination, respectively. The *D* test was used to test for inducible resistance to clindamycin. Susceptibility to three nonantibiotic compounds was tested with 6-mm discs impregnated with cadmium acetate (50 µg), mercuric chloride (109 µg), and ethidium bromide (50 µg) and *S. aureus* WBG248 was used as quality control strain [14].

Molecular Typing of *S. aureus* Isolates

DNA for staphylococcal protein A (*spa* typing) was isolated as described previously [15]. *spa* typing was performed on all isolates using protocol and primers published previously [16] and assigned to *spa* types using the *spa* typing website (<http://www.spaserver.ridom.de>). MLST was performed on all isolates as described by Enright et al. [17].

DNA Microarray Analysis

DNA microarray analysis was performed as described previously [18] to detect genes encoding species markers, SCC*mec*, virulence, and antibiotic resistance genes.

Results

DNA microarray analysis of 214 *S. aureus* obtained from patients in three different hospitals in Central Nigeria revealed that 22 of the isolates belonged to CC30-MSSA.

The 22 CC30-MSSA isolates were characterized further using antibiogram, *spa* typing, and MLST.

All 22 isolates were susceptible to vancomycin (MIC ≤2 µg/mL), teicoplanin (MIC ≤2 µg/mL), ceftiofloxacin, mupirocin, fusidic acid, erythromycin, clindamycin, gentamicin, kanamycin, trimethoprim, chloramphenicol, and rifampicin, but were resistant to penicillin G (*n* = 22; 100.0%), tetracycline (*n* = 12; 54.5%), and ciprofloxacin (*n* = 15; 68.2%). The isolates were also resistant to cadmium acetate (*n* = 22; 100%), ethidium bromide (*n* = 3; 13.6%), and mercuric chloride (*n* = 13; 59.1%).

The genetic determinants of the antibiotic resistance are summarized in Table 1. Penicillin resistance was mediated by *blaZ*, tetracycline resistance was mediated by *tet(M)*, while resistance to mercuric chloride was mediated by *merA* and *merB*.

Virulence Determinants of the CC30-MSSA Isolates

All 22 isolates were positive for genes that code for accessory gene regulator type 3 (*agr3*) and capsular polysaccharide type 8 (*cap8*). The isolates were also positive for genes encoding hemolysins (*hla*, *hly*, *hld*), biofilm synthesis proteins (*icaA*, *icaC*, *icaD*), serine protease E (*spIE*), clumping factors A/B (*clfA*, *clfB*), fibronectin-binding proteins A/B (*fnbA*, *fnbB*), collagen-binding adhesion (*cna*), enolase (*eno*), von Willebrand factor-binding protein (*vwb*), and immune evasion clusters including staphylokinase (*sak*), chemotaxis-inhibiting protein (*chp*), and staphylococcal complement inhibitor (*scn*).

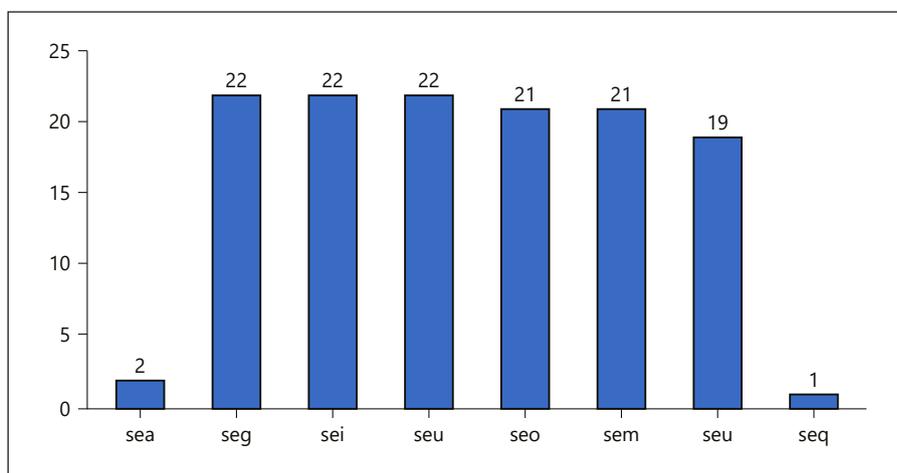


Fig. 1. Distribution of genes for staphylococcal enterotoxins.

All isolates were positive for genes for enterotoxin gene cluster (*egc*), which consists of enterotoxins *seg*, *sei*, *seu*, *seo*, *sem*, and *sen*. Three isolates lacked *seu*, while one isolate lacked *sem* and *seo*. Two isolates were positive for *sea*. The distribution of the SE genes is presented in Figure 1. Eight isolates were positive for *lukS-PV-lukF-PV* that code for PVL. The gene for epidermal cell differentiation inhibitors (*edinA*) and (*edinB*) was detected in one isolate. All 22 isolates were negative for *tst1* that codes for toxic shock syndrome toxin.

Molecular Characterization of CC30-MSSA Isolates

The CC30 isolates belonged to five *spa* types, t012 ($n = 12$; 54.5%), t019 ($n = 5$; 22.7%), t017 ($n = 2$; 9.1%), t037 ($n = 2$; 9.1%), and t318 ($n = 1$; 4.5%). MLST of the 22 isolates revealed that all belonged to the same sequence type, ST30. Twelve of the 22 ST30-MSSA isolates belonged to t012 (t012-/ST30-MSSA). The isolates were obtained from blood ($n = 6$), wound ($n = 4$), urethral swab ($n = 1$), and ear swab ($n = 1$). Ten of the t012 isolates were obtained from one hospital (PSSH), while each of the remaining two isolates was obtained from JUTH and BUTH. Eleven of the 12 isolates were resistant to tetracycline, ciprofloxacin, cadmium acetate, and mercuric chloride and harbored *tet(M)*, *merA*, and *merB* (Table 1). Eleven of the t012 isolates were positive for genes for clumping factors, biofilm production, enterotoxin gene cluster (*egc*), and immune evasion clusters. None of t012 isolates were positive for *lukS-PV-lukF-PV* and *tst1*.

Two isolates belonging to t017-CC30/ST30-MSSA were isolated from urine and wound samples in two hospitals (JUTH and PSSH). Both isolates were resistant to penicillin G, tetracycline, ciprofloxacin, cadmium ace-

tate, and mercuric chloride and carried *blaZ tet(M)* and *merA* and *merB*. One isolate was resistant to ethidium bromide. Both isolates were positive for *sea* and *egc*. One isolate lacked *sem* and *seu*.

Five isolates identified as t019-ST30-MSSA (PVL+) were obtained from blood ($n = 2$) and wound ($n = 3$) samples and belonged to *spa* type, t019. All five isolates were resistant to penicillin G mediated by *blaZ*. In addition, two of the isolates were resistant to cadmium acetate. All t019 isolates were positive for *lukS-PV-lukF-PV* (PVL) and *egc*.

Two isolates, identified as t037-ST30-MSSA (PVL+), were obtained from blood and wound samples of patients in the same hospital (JUTH). Both isolates were resistant to penicillin G, ciprofloxacin, cadmium acetate, and ethidium bromide. Both were positive for *lukS-PV-lukF-PV* (PVL). A single t318-ST30-MSSA (PVL+) isolate was isolated from a wound sample. It was resistant to penicillin G and cadmium acetate and was positive for *blaZ*. It was also positive for genes encoding epidermal cell differentiation inhibitors B and C (*edinB* and *edinC*), *lukS-PV-lukF-PV*, *egc*, and *seq*.

Discussion

This study investigated antibacterial resistance and virulence determinants in recent isolates of *S. aureus* belonging to CC30 (CC30-MSSA) obtained from three tertiary hospitals in North-Central Nigeria. *S. aureus* isolates belonging to CC30 have had significant impacts on human infections since it was first identified as phage-type 80/81 in the 1950s [2–5]. The pandemic *S. aureus* phage

80/81 clone caused major outbreaks of infections in the 1950s and early 1960s in several countries including Australia, England, USA, and Canada [4, 5, 19]. The isolates of the phage 80/81 clone later acquired methicillin resistance (*mecA*) and evolved into the Southwest Pacific clone of community-associated MRSA (ST30-MRSA-IV [PVL⁺]) and the healthcare-associated MRSA clone (ST36-MRSA-II) [2, 7, 8]. Interestingly, the prevalence of the pandemic phage 80/81 strains declined with the increase in the prevalence of the CC30-MRSA clones [2]. However, there are recent reports of CC30-MSSA in some countries including Ecuador [9], Lebanon [10], Greece [11], Canada [19], USA [20, 21], and Germany [22]. The CC30-MSSA isolates in this study add to the growing list of recent reports of this lineage. The recent reports of the CC30-MSSA isolates may represent a re-emergence of the pandemic phage 80/81 clone or the emergence of CC30-MRSA isolates that have lost *mecA* that codes for methicillin resistance. The reports may also show increasing awareness of CC30-MSSA as significant human pathogens [11, 19–23].

All 22 CC30-MSSA isolates in this study were resistant to penicillin G mediated by *blaZ*, with 54.5% of them resistant to tetracycline mediated by *tet(M)*, which is comparable to the resistance characteristics of the phage group 80/81 isolates [2, 5, 24]. In addition, 68.2% of our isolates were resistant to ciprofloxacin, an antibiotic that was not available in the 1950s, indicating recent acquisition of ciprofloxacin resistance by these isolates. In contrast, a recent study conducted in Poland revealed that all *S. aureus* phage 80/81 strains obtained from patients with oral infection were sensitive to ciprofloxacin [25]. Resistance to tetracycline and ciprofloxacin has also been reported in ST30-MRSA isolates [15, 24, 25]. Although the isolates in this study were susceptible to erythromycin and clindamycin, 71% and 63% of CC30-MSSA causing ocular infections in patients in the USA were resistant to erythromycin and clindamycin, respectively [21]. The differences in the susceptibility patterns may reflect exposure to different antibiotics at the different clinical units or hospitals.

The 22 CC30-MSSA isolates were all resistant to cadmium acetate, while 59.1% of them were resistant to mercuric chloride. *S. aureus* strain, ATCC25923, a pre-pandemic phage 80/81 strain traditionally used as quality control strain for antibiotic susceptibility testing of *S. aureus*, has been reported to harbor a plasmid-mediated cadmium resistance [14], indicating that *S. aureus* acquired cadmium resistance prior to penicillin resistance.

The 22 isolates in this study belonged to sequence type 30 (ST30), accessory gene regulator type 3 (*agrIII*), and

capsular polysaccharide type 8 but were heterogeneous in their *spa* types. The dominant *spa* type was t012 (12/22) followed by t019 (5/22), t017 (2/22), t037 (2/22), and t318 (1/22). Similarly, t012 was the dominant *spa* type among CC30-MSSA nasal colonizers in Germany [22] and among ST30-MSSA isolates in Lebanon [10]. The other *spa* types, t017 and t019, have also been associated with ST30-MSSA and ST30-MRSA isolates [15, 20, 22]. Interestingly, two of the isolates belonged to t037 that is usually associated with ST239-MRSA-III isolates [26]. ST239 isolates are considered to be mosaic strains formed by the recombination between ST8 and ST30 parents [7] but have *spa* types typical of ST30 strains [22], suggesting that the t037 isolates in this study are closer to ST239. This is supported by their multiresistance to ciprofloxacin, cadmium acetate, mercuric chloride, and ethidium bromide, which is a typical resistance pattern of ST239-MRSA isolates [26].

The isolates were positive for a variety of virulence-related genes. Eight of the 22 CC30-MSSA isolates comprising t019 (*N* = 5), t037 (*N* = 2), and t318 (*N* = 1) were positive for *lukF-PV-lukS-PV* that encodes PVL similar to the phage 80/81 that evolved into the Southwest Pacific MRSA clone [7, 8]. Similarly, Gomes et al. [27], who investigated *S. aureus* strains collected in Denmark between 1957 and 1973, found that two of three t012 isolates in their study were positive for PVL.

All 22 isolates were positive for *agrIII*, *cap8*, *clfA*, *clfB*, *icaA*, *icaC*, *icaD*, and *cna* as has also been reported in ST30-MRSA isolates [10, 15, 28]. The enterotoxin gene cluster (*egc*) was common among the ST30-MSSA isolates. However, *sea* was only detected in the t017 isolates, while *seq* was detected in a single isolate.

None of the isolates were positive for *tstI* that encodes toxic shock syndrome toxin. In contrast, other studies conducted in different parts of the world have reported the carriage of *tstI* in ST30 isolates [11, 15, 22, 29]. In addition, ST30-MRSA isolates recently emerged in Kuwait and Saudi Arabia that carried SCC*mec* type VI with fusidic acid resistance and *tstI* [30], suggesting that *tstI* has recently been acquired by ST30-MRSA in the Arabian Gulf.

Limitations of this study include the small number of isolates investigated and the absence of phage typing data on the isolates. Nevertheless, the data presented draw attention to the presence of CC30-MSSA isolates that possess phenotypic and molecular characteristics that are comparable to those of the phage 80/81 pandemic clone in an area where little is known about the existence of CC30-MRSA strains.

Conclusion

This study has provided insights into the epidemiology of ST30-MSSA isolates recovered from healthcare facilities in Jos, Nigeria. The ST30-MSSA were isolated mostly from wound and blood infections, were resistant to penicillin G, and harbored *blaZ* and the enterotoxin genes cluster (*egc*), which were comparable to the characteristics of the pandemic phage-type 80/81 strain that were reported in the 1950s and early 1960s. Further surveillance studies are recommended to monitor the changing trends in epidemiology of *S. aureus* causing healthcare and community-associated infections for better patient management, prevention, and control of infection.

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Statement of Ethics

This study was approved by the Ethical Committees of Jos University Teaching Hospital, Jos (approval No. JUTH/DCS/ADM/127/XXV/314), Plateau State Specialist Hospital, Jos (ap-

proval No. PSSH/ADM/ETH.CO/2017/006), and Bingham University Teaching Hospital, Jos, Plateau State (approval No. NHREC/21/05/2005/00495).

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Author Contributions

Conception and design of the study were contributed by Edet E. Udo. Methodology, acquisition, and analysis of data were carried by Samar S. Boswihi and Unyime C. Essien. Supervision of the study was contributed by Edet E. Udo and Nneka R. Agbakoba. Writing – original draft was carried by UCE, and writing – review and editing the manuscript were carried by Edet E. Udo, Unyime C. Essien, Samar S. Boswihi, and Nneka R. Agbakoba.

Data Availability Statement

All data generated during this study are included in this paper.

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