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Towards Understanding the Clonality of Methicillin Resistant *Staphylococcus Aureus* in Sokoto State Nigeria

Shuaibu Suleiman Adeiza *, Josiah Ademola Onaolapo, Busayo Olalekan Olayinka

Department of Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria

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***Corresponding author:**
Shuaibu Suleiman Adeiza. Department of Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria

Phone: +8162208444

Fax: +8162208444

Email:
shuaibusuleiman60@yahoo.com

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Abstract

Introduction: The epidemiology of MRSA strains is constantly changing and the potential utility of genomic surveillance in defining its population structure and outbreaks of possible high-risk lineages in Sokoto state cannot be overstated. This study brings to light the various clones, *spa*-types, sequence types (ST) and the resultant clonal complexes (CC) of MRSA strains in Sokoto state as a step towards an all-inclusive MRSA surveillance system.

Methods: Phenotypic determination of the presence of MRSA was carried out by subjecting the isolates to Oxacillin resistance screening agar base test (ORSAB). The PCR products (*mecA* and *spa*) where electrophoresed on 1.5% agarose gel and the *spa* positive isolates were sequenced and analyzed. Based upon repeat pattern (BURP) algorithm was used to compute *spa* clonal complexes (*spa*-CCs)

Results: In this study, analysis of 16 MRSA strains revealed 10 different *spa*-types, varying in length between 1 (t267, t021), 2 (t044, t127) and 4 (t1839, t202) repeats, which were clustered into 2 different groups and 2 singletons. The MLST mapping of the *spa* types of 16 MRSA isolates revealed seven different sequence types (ST-1, ST-9, ST-55, ST-93, ST-97, ST-80 and ST-463) clustered into spa-CC 07and spa-CC 003.

Conclusion: The present study provides an understanding of the MRSA clonal population in Sokoto, revealing the 10 different *spa*-types in circulation and providing awareness of the proliferation of s*pa*-types t091 and t044. Genetic Surveillance for these clones needs to be coordinated nationally and locally because successful clones are not confined by state or country boundaries.

Keywords: *Spa*-Types, Clonal Complexes, Sequence Types, MRSA, Sokoto

Introduction

The lack of restraint in antibiotics prescription and its overuse in healthcare and livestock growth promotion has provoked a gradual but steady build-up of antimicrobial resistance(1). Poor hygiene and sanitation have also contributed to rise in antimicrobial resistance by facilitating its spread (2). Antibiotics steered selective pressure on *Staphylococcus aureus* (*S. aureus*) has led to the evolution of methicillin resistant *S. aureus* (MRSA) over the years. This pathogen causes various skin, soft tissue and systemic infections(3). The prevalence of MRSA varies from one geographical location to another with different pandemic origin (4). MRSA lineages are based on clonal complexes (CC); group of sequence types (ST) with at least 5 out of 7 identical alleles (5). Genetically similar (clonal) MRSA strains may differ in their pathogenic potential due to the presence or level of expression of the virulence factor (6). The cell walls and cytoplasm of MRSA house a macromolecule called Staphylococcal Protein A. This protein

of 42D molecular weight is encoded by the *spa* gene and comprises of short sequence repeats (SSR) variations that are related to Staphylococcal pathogenesis and virulence (7). The nucleotide sequence of this protein contains repeated parts positioned at 3' end known as the X region and consist of 12 units each with a length of 24 highly polymorphic nucleotides (7). Generally, *spa*-types can be mapped to identified MLST designations (8) and this study takes advantage of this facility owing to the multifunctional capacity of the Ridom spa server. In our study location (Sokoto) the clonality of MRSA is largely shrouded in mystery. This study brings to light for the first time, the various clones of MRSA, *spa*-types and corresponding sequence types and clonal complexes (CC).

Methods

The ethical review board of the Sokoto state ministry of health approved this research (SMH/1580/V. IV) after a successful defense of the research proposal. Prospective participants were informed of the details of the study and assured of anonymity. Only participant who consented to participate in the study were eventually enrolled. For bacterial isolates, a total of forty-three (43) *S. aureus* isolates were obtained from nasal swabs of patients and hospital staff of Sokoto State-owned Hospitals (Specialist Hospital Sokoto, SPH, Maryam Abacha women and children hospital, MRY and Orthopedic hospital Wamakko, WMK) in the year 2018. The isolates were identified phenotypically by standard methods such as Gram staining, catalase, coagulase tests and Microgen: Staph ID kit. Phenotypic determination of the presence of MRSA was carried out by subjecting the isolates to Oxacillin resistance screening agar base test (ORSAB). The emergence of bluish colonies from overnight culture plates were indicative of methicillin resistance. MRSA genomic DNA was extracted from an overnight culture in nutrient agar using the Qiagen DNA extraction kit in accordance with the manufacturer's protocols. Carriage of Methicillin resistance determinant and Protein A were determined based on presence of *mecA* (533bp) and *spa* (variable bp) genes, using designed primers; *mecA*; Forward-TGGTAAAGGTTGGCAAAAAGA, Reverse-TTGTCCGTAACCGGAATCA (Accession number KY788636), Spa; Forward-CTCAAGCACCAAAAGAGGAAG, Reverse-ATGTACTCCGTTGCCGTCTT (Accession number; EF203507). The primers were calculated with Primer-3-plus® and sent to Indaba Biotec South Africa for production. In order to characterize the nucleotide sequences by gel bands, the PCR products where electrophoresed on 1.5% agarose gel. After band visualization (BIO-RAD™ gel doc, Milan, Italy), the products were purified (Qiagen™ clean up kit) and sequenced. The *spa* types were allotted via the Ridom Staph Type software version 1.4 (Ridom, Germany) and the Ridom Spa Server(9). Based upon repeat pattern (BURP) algorithm was used to compute *spa* clonal complexes (*spa*-CCs). There were no exclusion concerning number of repeats; cost less or equal to 4; a cluster that housed 2 or more related *spa*-types was considered as CC; a *spa*-Type that was not gathered into a CC was referred to as a singleton. A Minimum spanning tree depicting the *spa* diversity among MRSA strains originating from Nasal swabs was figured using PHYLOViZ (2).

Results

Out of 43 phenotypically confirmed *S. aureus* isolates cultured on ORSAB medium, 38 (88.4%) strains exhibited phenotypic methicillin resistance. Only 16 (42.1%) of these strains harboured the *mecA* gene. Eighteen (47.4%) strains were found positive for the *spa* gene (Figure 1). The *spa* sequences were deposited with GenBank under the accession numbers MK690483, MK690484, MK690485, MK690486, MK690487, MK690488, MK690489, MK690490, MK690491, MK690492, MK690493, MK690494, MK690495, MK690496, MK690497 and MK690498. The *spa* typing

analysis revealed 10 distinct *spa*-types within the group of 16 strains. The *spa* repeats sequences specific for each known *spa*-type is shown in Table 1 with the most prevalent *spa*-type (t091) accounting for 3 out of 13 typed strains (23.1%). It was followed by t044, consisting of 2 strains (15.4%). All the other 8 *spa*-type housed only 1 strain each. Three sequences that were likely *spa* repeat, but did not exist in Ridom database were considered as 'unidentified' *spa*-type. About 18.75% of sequence repeats were evidenced as unknown spa types (Figure 2). Figure 3 shows a minimum spanning tree portraying genotypic assortment among MRSA isolates from nasal swabs. Each node in this minimum spanning tree typifies the *spa*-type of a single MRSA isolate. Nodes with identical colours represent isolates from the same hospital. The lines connecting the isolates denotes their genetic distances. It was observed in the figure that the isolates were closely linked with each other. Most of the *spa*-types share a common lineage with type t267. The *spa* attributes were ascribed to clonal complexes and sequence types by mapping in Ridom *spa* server (Table 1). A total, 4 clonal complexes were mapped out. Even though the number of clonal complexes mapped out were few, it remained instructive to carry out a BURP analysis to infer the relatedness of the included isolates (Figure 4). The isolates included in the analysis segregated in two main clonal complexes, *spa*-CC007 (9 strains) and *spa*-CC 003 (2 strains), and two singletons.

Table 1: Distribution of spa types with associated spa-CC and Sequence types (ST) mapped from Ridom *spa* server.

Sample ID	Repeat units	Repeat sequence (Ridom)	*spa*-types	ST	*spa*-CC
SPH004R	7	r07:r23:r12:r34:r34:r33:r34	t044	ST-80	CC-07
SPH015R	10	r26:r23:r13:r25:r17:r34:r34:r34:r33:r34	t1839	ST-463	CC-06
SPH029L	8	r07:r23:r21:r16:r34:r34:r33:r13	t098	ST-1	CC-07
SPH038L	8	r08:r16:r02:r16:r02:r16:r17:r24	*	*	*
SPH045L	7	r07:r23:r21:r16:r34:r33:r13	t127	ST-1	CC-07
SPH086R	9	r15:r12:r16:r02:r16:r02:r25:r17:r24	t021	ST-55	CC-03
SPH103R	9	r08:r16:r02:r25:r02:r25:r34:r24:r25	t034	ST-55	CC-03
WMK024L	8	r11:r17:r23:r17:r17:r16:r16:r25	t202	ST-93	CC-02
WMK024R	9	r07:r16:r12:r23:r02:r02:r34:r34:r34	*	*	*
MRY053L	10	r07:r23:r12:r21:r17:r34:r34:r34:r33:r34	t267	ST-97	CC-07
MRY082R	7	r13:r12:r17:r17:r23:r18:r17	*	*	*
MRY124R	10	r07:r23:r21:r17:r34:r12:r23:r02:r12:r23	t091	ST-1	CC-07
WMK026R	10	r07: r23:r21:r17:r34:r12:r23:r02:r12:r23	t091	ST-1	CC-07
WMK031R	7	r07:r23:r12:r34:r34:r33:r34	t044	ST-80	CC-07
WMK053R	10	r07:r23:r21:r17:r34:r12:r23:r02:r12:r23	t091	ST-1	CC-07
WMK119R	10	r07:r16:r12:r23:r02:r12:r23:r02:r02:r34	t2700	ST-9	CC-07

* indicates a sequence that is a likely spa repeat, but does not exist in Ridom database

Key: CC= Clonal Complexes, ST= Sequence types (MLST types)

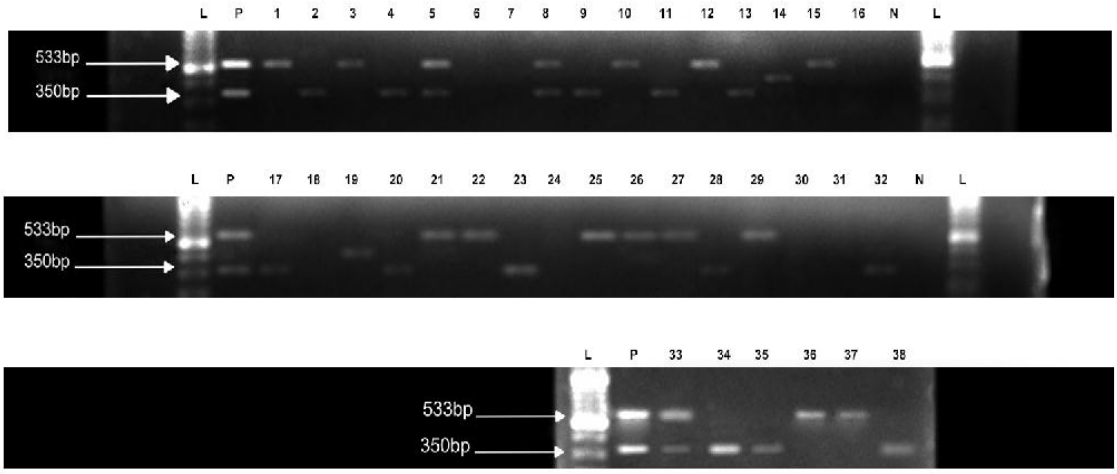

Figure 1. Electrophoretogram of ethidium bromide stained gel of 38 MRSA isolates showing amplification multiplex PCR products of *mecA* (533bp) and *spa* (350bp - 433bp) gene. In the figure L is 100 bp plus DNA ladder. P and N represents positive and negative controls.

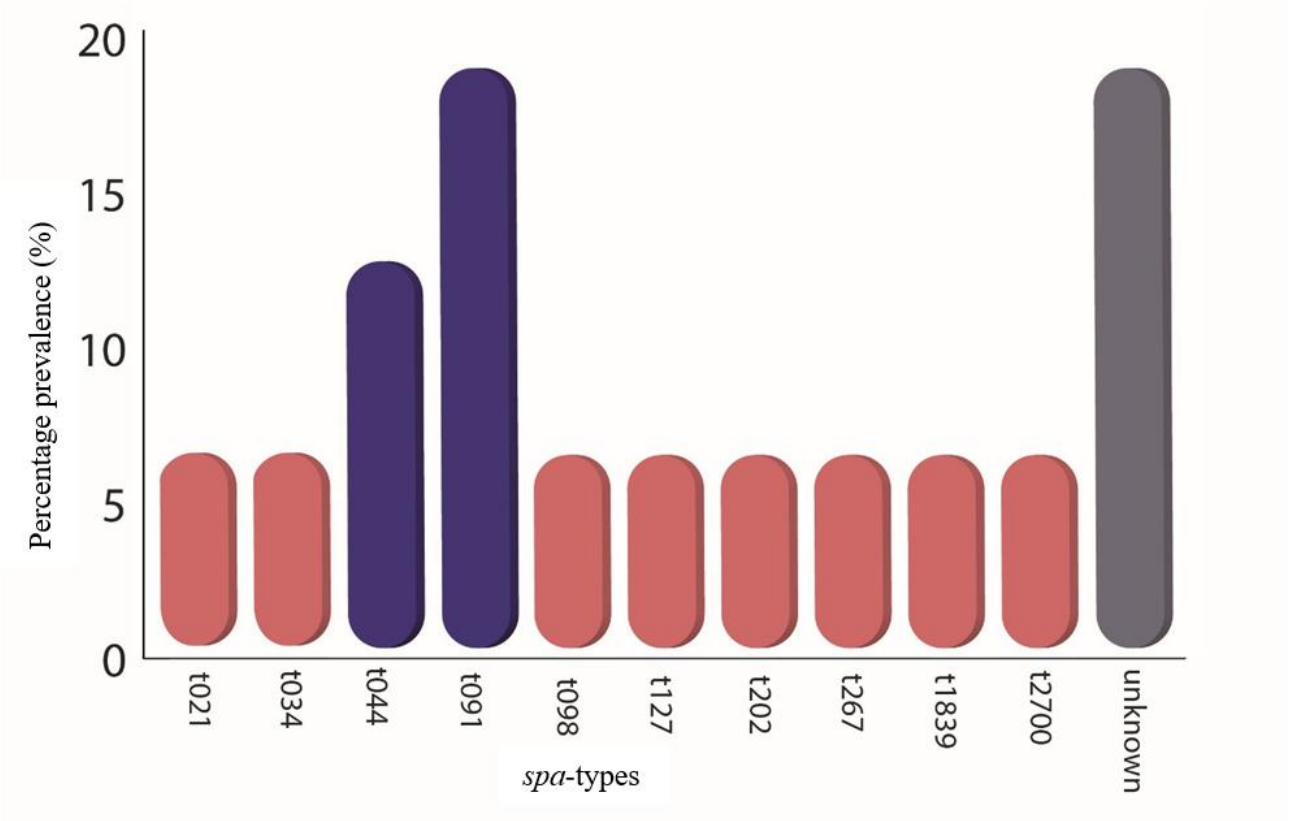

Figure 2. Distribution of *spa*-types among MRSA isolates from Sokoto state

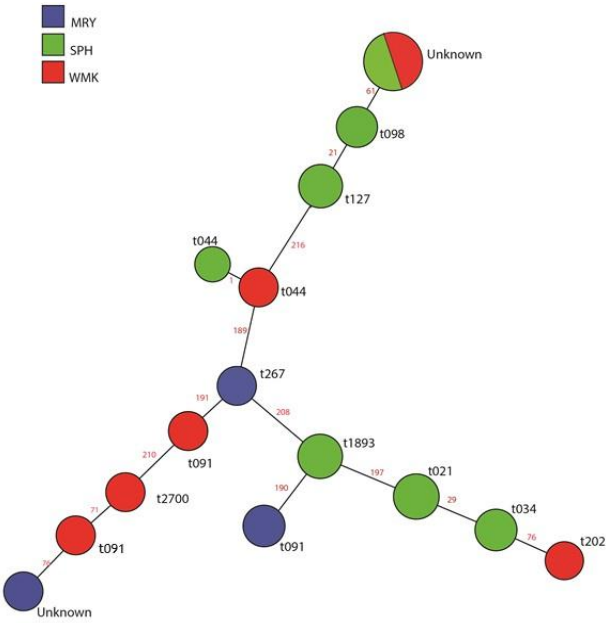

Figure 3. Minimum spanning tree showing the relationship of the Hospital isolates and spa type determined using PHYLOViZ: the different colours represent the hospitals, namely green for Specialist Hospital Sokoto (SPH), red for Orthopedic Hospital Wamakko (WMK) and blue for Maryam Abacha women and Children Hospital (MRY). Each circle represents one *spa*-type, the size of which is related to the number of strains within the *spa*-type.

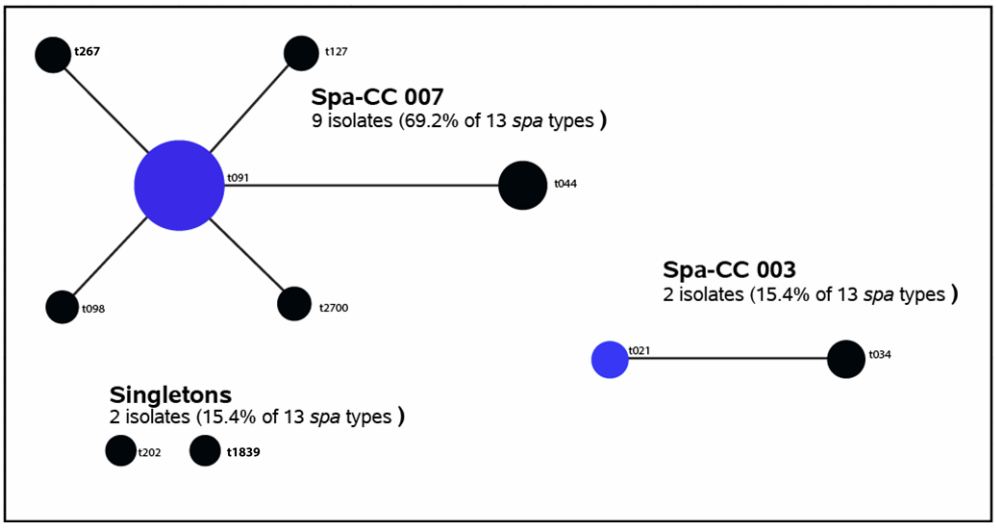

Figure 4. Based upon repeat analysis (BURP analysis with a cost of 4) showing the population structure of 13 MRSA isolates. Clusters of linked spa types correspond to *spa* clonal complexes (spa-CCs). The *spa* types indicated in blue were defined as founders of particular clusters. In total, two spa-CCs have been identified, with spa-CC 07and spa-CC 003 accounting for 11 of 13 isolates. Two spa types were regarded as singletons.

Discussion

Protein A is an anti-phagocytic shield that hinders *S. aureus* ingestion by neutrophils; it plays a key role in the virulent nature of the organism (8). This protein is coded by *spa* gene and identifies as the X region. The nucleotide sequence of the protein is made up of repetitive regions called "repeats". Each repeat is about 24 nucleotides long and form the basis for *spa*-sequence typing (10). *spa*-typing was used in this study to determine the population structure of MRSA because of the high level of polymorphism within the nucleotide sequence of the X region of the *spa* gene and the fact that *spa* typing is associated with speed, low costs, and comparability of data (11). The present study reports that although 88.4% (38/43) of *S. aureus* were phenotypically confirmed as MRSA, the *spa* gene was not visualized in 52.6% (20/38) of MRSA isolates. Conversely, only 47.4% of the strains harboured the *spa* gene. Compared to the works of (10) in Iran who reported an absence rate of 3.8%, the low *spa* prevalence may be explained by misidentification as a result of mutation in the *spa* nucleotides and natural strain dynamics(12). The presence of *mecA* gene is a standard for MRSA diagnosis. According to this study 42.1% of 38 phenotypically confirmed methicillin resistant isolates harboured *mecA* gene. The prevalence of MRSA observed here could be associated with MRSA cross-transmission and the effect of antibiotics selective pressure on circulating strains (13). There is evidence that MRSA strains can regain their methicillin susceptibility by spontaneous excision of the *mec* element (14). We noticed in this study that *mecA* bands were seen in only 22.2% of the *spa* positive strains nevertheless *spa* typing revealed that the strains had ancestries from MRSA strains. It is therefore possible that some of these strains may have lost their *mec* elements. In this study, analysis of MRSA isolates revealed ten (10) different spa types, varying in length between 1 (t267, t021), 2 (t044, t127) and 4 (t1839, t202) repeats, which were clustered into 2 different groups and 2 singletons by BURP analysis. The reassortment of repetitive sequences caused by the repair of double-strand breaks during DNA replication may be responsible for the spa diversity observed in this study (15). To our knowledge, this is the first report of t091, t044, and t127 *spa*-types in Sokoto. The dominant *spa*-type in this study was t091 and accounted for 3 out of 13 of the typed strains (23.1%), this dominance is corroborated by a 17% prevalence reported by (16) in a southern Nigerian study. Contrasting results on the inconsistency of the X region of the *spa* gene have been reported (17), (18). Even though Population structures are influenced by regional and sample variations, the type of structures found in this study were similar to the works of (19) in Germany and (20) in Algeria, where the most common *spa* type was t091 (6.1%). The finding of *spa* type t044 (12.5%) as the second most common after type t091(18.75%) corroborated an earlier report in Riyadh (21) where has also been reported as second most prevalent *spa*-type. Studies have revealed that the frequency of genetic variation in short sequence repeats (SSR) in studies conducted under laboratory conditions (without selective pressures) are sometimes low compared to in vivo studies where as much as 10% mutations within the polymorphic X region of the *spa* gene has been observed (22), (11). Another possible clarification for the dominance of *spa* type t091 in this study is that this strain harbours some virulence factors facilitating its spread. The vast number of *spa* types and the hypervariability of the SSR region typed mandated more classification into *spa* complexes in terms of similarities of SSR unit (22). The distribution of analogous *spa* types into *spa* complexes aids the understanding of novel, emerging *spa* types. Most of the strains (69.2%) belonged to *spa*-CC 007 and majority were hospital acquired. A study conducted by (23) showed that any 2 *spa* types with identical repeats or repeats that differed in a single deletion or insertion of the nucleotide sequence fit into the same cluster. The *spa*

types t091, t098 and t127 differ only in the 2-*spa* repeat and were all assigned to the same BURP group. It is well established that the prevalence of different *spa*-types varies based on the geographic area and can change over time (13). In order to understand the directionality of spread of the strains amongst the hospitals, the relationship between *spa* types was visualized in a minimum spanning tree that revealed a diverse but also highly clonal MRSA population structure. It can be inferred that the *spa* type t267 from Maryam Abacha Women and children Hospital shared a central phylogenetic relatedness with all MRSA isolates obtained in this study. This may not be unconnected to the fact that the Hospital is situated very close to one of the largest markets in the state and Patients from diverse ethnicities visit the hospital and come in contact with people from the market and hence importing the diversity detected. MLST mapping (Ridom server) revealed that *spa*-t267 matched excellently with ST-97 (MRSA) which originated from the United Arab Emirates isolated in 2015. MRSA ST-97 was first recorded in Denmark (2004), South Africa (2013,14and 15) and now in Sokoto, Nigeria(9). The MLST mapping of the *spa* types of 16 MRSA isolates revealed seven different sequence types (ST-1, ST-9, ST-55, ST-93, ST-97, ST-80 and ST-463). The proliferation of ST1 and ST80-MRSA in this study is consistent with trends in Africa (Nigeria, Egypt, Algeria and Tunisia). Overall, the clonal distribution of MRSA obtained in this study is consistent with results reported in a number of studies(24), (25), Studies have suggested that certain MRSA clones are more successful than others at colonizing, surviving, and spreading at different geographical location (25). Previous studies have shown that strains of ST1 and ST80 both carry the Panton-Valentine leucocidin gene and SCCmec IV (26). Thus, there might be a phylogenetic association of these spa types within CC07 that is reflected by a related SSR structure, although the MLST is different. The typing of clinically relevant MRSA-type ST1-t127 known to harbour both *mecA* and *mecC* gene on the same SCCmec in this study is noteworthy. ST1-t127 MRSA strains are community acquired and are often associated with street urchins and drug addicts (27). We also documented a single clone of MRSA *spa*-type t267/ST-97 in our sampling frame, this clone houses 0.33% of the Ridom database strains of which more than 48% are classified as MRSA. This further supports its association with MRSA and MSSA globally (28,29). A previous study by (30) revealed *spa* CC-07 as one of the most epidemic MRSA cluster worldwide. The group is largely associated with hospital and community acquired MRSA (HA-MRSA and CA-MRSA) (31) . Therefore, its predominance in this study is not surprising. Furthermore, *spa*-CC03 as reported by (5) shares lineages with German clones and livestock stock associated MRSA (LA-MRSA). This suggests the transmission of these clones between humans and animals. Interestingly, three (18.75%) of the studied MRSA strains had known repeats but the sequence did not match any of the 19,163 *spa* types registered in the database. This may have occurred as a result of the polymorphic nature of the X region of the *spa* protein. The random arrangement of these repeats during a recombination event may have generated new *spa* types due to DNA polymerase error, deletion, or duplication events. (32).

Conclusion

The present study provides an understanding of the MRSA clonal population in Sokoto, revealing the 10 different *spa*-types in circulation and providing awareness of the proliferation of *spa*-types t091 and t044. We documented ST1, ST55 and ST80-MRSA the prevalent sequence types. Most of the clones belonged to two clonal complexes (CC); *spa*-CC 007 and *spa*-CC 003. The study also emphasizes presence of unknown spa-types signifying the need to be vigilant to identify emergence of new MRSA strains. Genetic Surveillance for these clones needs to be coordinated nationally and locally because

successful clones are not confined by state or country boundaries.

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Ethical issues

Not applicable.

Authors' contributions

The Conceptualization and supervision of this work was accomplished by Onaolapo J.A and Olayinka B.O. Investigation, sequence analysis and writing were executed by Adeiza S.S. All authors contributed to and approved the final manuscript.

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