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The Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) of *Staphylococuss aureus* clinical isolates recovered from the Provincial Specialist Hospital in Lublin, Poland

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ABSTRACT

Understanding the epidemiology and evolution of *Staphylococcus aureus* (SA) is crucial for implementing effective infection prevention and control measures. In this study, a short-term (4-month) and local molecular epidemiology of SA in the nosocomial setting was investigated using multiple-locus variable number tandem repeat (VNTR) analysis (MLVA). The study included 31 clinical SA isolates. The analysis was based on two complimentary panels encompassing 14 VNTR loci wherein a first-line assay comprised a subset of 10 loci (Panel 1), followed by a second subset of four loci (Panel 2). Thirty MLVA profiles were generated. Small amplicons were obtained for three out of the four loci included in Panel 2 for more than half of the isolates, making the calculation of the number of repeats impossible. Therefore, phylogenetic clustering was based on the analysis of basic loci included in Panel 1 (which identified two major genetic clusters). Cluster I included 26 (84%) isolates, among which 14 were classified into five sub-clusters (cutoff value ≥65%). Only one pair of the isolates shared an identical MLVA pattern. Our study indicates the usefulness of a MLVA tool comprised of a primary subset of 10 loci to track the possibility of SA intra-hospital transmission. We report a high genetic diversity of the isolates and suggest a limited degree of intra- and inter-ward SA transmission. Further genetic studies are necessary to investigate the nature of the small alleles yielded in Panel, as their subset can provide even higher resolution of the assay.

INTRODUCTION

Staphylococcus aureus (SA) is one of the most common opportunistic human pathogens involved in the etiology of community- and hospital-acquired diseases, exemplified by skin and soft tissue infections, endocarditis, osteomyelitis, bacteremia, pneumonia and toxic shock syndrome. Disruption of the integrity of the skin and mucous membranes due to surgical procedures, wounds, or chronic skin conditions are among the most important factors predisposing to SA invasion. Additionally, young and an advanced age, immunosuppression, as well as administration of medical devices account for a greater vulnerability to infections of this etiology [1,2]. In addition to a plethora of virulence factors involved in tissue invasion, cytotoxicity and intracellular persistence, increasing prevalence of antibiotic resistance in SA has contributed to classification of this microorganism as a major threat to public health [3,4]. Methicillin-resistant SA (MRSA) strains, resistant to most members of the β-lactam family of antibiotics, have emerged as important pathogens in nosocomial, community and veterinary settings [4,5]. Particularly, the occurrence and transmission of MRSA in the hospitals, where these strains can be endemic, have been associated with more difficult-to-treat infections and increased mortality rates [5]. Understanding the epidemiology and evolution of SA is of the uppermost significance to implement effective infection prevention and control measures in order to prevent microbial transmission to vulnerable patients and healthcare workers [5,6].

Many molecular typing methods based on the analysis of the polymorphism at the level of bacterial DNA are available to investigate outbreaks and track SA transmission, both at the local and global levels. Tandemly repeated genomic sequences provide a valuable source of the DNA polymorphism, and multiple-locus VNTR (variable-number tandem-repeat) analysis (MLVA) has been considered as the most useful assay among the PCR-based genotyping approaches

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[6,7]. The strain genotype is presented as a sequence of numbers corresponding to the number of repeats at each analysed locus which can be readily incorporated into large databases and used for epidemiological studies [8]. Along with the Pulse Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST) and Staphylococcal Protein A (spa) typing, MLVA has been used for local and international large-scale investigations of SA molecular epidemiology. Of note, MLVA assay has been reported to demonstrate comparatively high or even greater discriminatory power when compared to the above mentioned typing methods [8-13]. Additionally, MLVA is rapid, inexpensive and easy to interpret, which makes it applicable for molecular typing in many laboratories. Moreover, its interlaboratory reproducibility enables the development of international databases [6,13].

The aim of the study was the MLVA-based short-term and local analysis of molecular epidemiology of SA. The study included clinical SA isolates recovered from the Provincial Specialist Hospital in Lublin, Poland.

Table 1. Characterization of SA isolates included in the study

Isolate no. n=31	Origin of the isolate		Antibiotic resistance profile
	Hospital ward	Clinical specimen	
01	Intensive Care Unit	Bronchoalveolar lavage	Inducible MLSB
02	Vascular Surgery Unit	Wound	Inducible MLSB
03	Internal Medicine Unit	Wound	Fully susceptible
04	Internal Medicine Unit	Ulcer	Fully susceptible
05	Alergology and Lung Diseases Unit	Urine	Fully susceptible
06	Alergology and Lung Diseases Unit	Blood	Fully susceptible
07	Cardiology Unit	Wound	Fully susceptible
08	Orthopedics Unit	Bursal fluid	Inducible MLSB
09	Intensive Care Unit	Blood	Inducible MLSB
10	Laryngology Unit	Wound	Streptogramins
11	Nephrology Unit	Blood	Constitutive MLSB
12	Toxicology Unit	Blood	Clindamycin
13	Internal Medicine Unit	Blood	Fully susceptible
14	Intensive Care Unit	Blood	Inducible MLSB
15	Toxicology Unit	Pleural effusion	Clindamycin
16	Toxicology Unit	Bronchoalveolar lavage	Inducible MLSB
17	Nephrology Unit	Blood	MRSA, Inducible MLSB
18	Intensive Care Unit	Peritoneal fluid	Inducible MLSB
19	Intensive Care Unit	Blood	Inducible MLSB
20	Intensive Care Unit	Bronchoalveolar lavage	Inducible MLSB
21	Neurology Unit	Bronchoalveolar lavage	Erythromycin
22	Surgery Unit	Wound	Inducible MLSB
23	Intensive Care Unit	Bronchoalveolar lavage	MRSA
38	Nephrology Unit	Blood	Streptogramins
39	Nephrology Unit	Blood	Inducible MLSB
41	Nephrology Unit	Blood	Fully susceptible
42	Nephrology Unit	Blood	Fully susceptible
43	Internal Medicine Unit	Wound	MRSA, Constitutive MLSB
45	Gastrology Unit	Blood	MRSA, Constitutive MLSB
46	Orthopedics Unit	Bronchoalveolar lavage	MRSA
47	Intensive Care Unit	Blood	Fully susceptible

MATERIALS AND METHODS

The study included 31 clinical SA isolates collected in the Provincial Specialist Hospital of Stefan Kardynał Wyszyński in Lublin, Poland, during a 4-month period (July-October 2022) in a passive surveillance. One isolate per a patient was subjected to genotyping. Information regarding types of clinical samples from which SA isolates were cultured and hospital wards in which the infected patients were hospitalized is included in Table 1. The majority of isolates were cultured from patients hospitalized in the Intensive Care (n=8; 25%), followed by Nephrology (n=6; 19%) and Internal Medicine (n=4; 13%) Units. As many as 14 (45%) SA isolates were cultured from blood, followed by wounds and bronchoalveolar lavage (n=6; 19%) isolates each for the two types of the specimens). Single isolates were cultured from urine, bursal fluid, pleural effusion and peritoneal fluid.

Identification of bacterial isolates to the species level and their antibiotic susceptibility testing were performed in the hospital laboratory using the BD Phoenix™ (Becton Dickinson, US) automatic system. Antimicrobial susceptibility of the isolates was interpreted according to recommendations of the European Committee on the Antibiotic Susceptibility Testing (EUCAST, v.12.0). Five (16%) SA isolates were MRSA; three of them demonstrated an additional macrolide-lincosamide-group B streptogramins (MLSB) resistance phenotype. The remaining SA isolates showed MLSB resistance (n=11) only, or were resistant to clindamycin (n=2), streptogramins (n=2) or erythromycin (n=1). Nine (29%) isolates were fully susceptible to the tested antimicrobials.

Nucleic acid extraction

Genomic DNA was extracted from pure 24-h Columbia blood agar SA cultures, using the Genomic Micro AX *Staphylococcus* Gravity kit (A&A Biotechnology, Poland) and following the manufacturer's protocol. The nucleic acid quality and concentration were analysed via BioTek Synergy LX spectrophotometer (Agilent Technologies, US).

Genotyping data production and analysis

The MLVA genotyping scheme was adopted from the study of Pourcel *et al*. [8]. The MLVA-14 assay included two complimentary panels encompassing a total of 14 VNTR loci. The 14 loci were analyzed in two steps, including a first-line assay comprising a subset of 10 loci (panel 1) and a second subset of 4 loci (panel 2) [8]. The MLVA was run with individual PCRs and agarose gel electrophoresis of amplicons for a subset of VNTRs.

Oligonucleotide sequences of VNTRs employed in this study are available in the source publication [8]. The PCR reactions were performed using the *Taq* PCR Core Kit (Qiagen, Netherlands). The 20-µl PCR master mix sample included 1×CoralLoad Buffer containing 1.5 mM $MgCl_2$, 1×Q Solution, 0.5 U *Taq* DNA polymerase, 200 µM of each deoxynucleoside triphosphate, 0.3 µM of each flanking primer (Genomed, Poland) and 2 µl of the DNA. The Q Solution was added to improve the melting behavior of a template DNA demonstrating a high degree of GC-contents – as previously reported for STAR genetic elements as applied in the MLVA assay (8). Amplification was performed

with a SensoQuest thermocycler (Labcycler) under the following conditions: initial denaturation cycle for 3 min at 94°C, 40 cycles of denaturation for 30 s at 94°C, annealing for 40 s at various temperatures depending on the VNTR locus [8], elongation for 60 s at 72°C plus a final elongation step for 10 min at 72°C.

The PCR products were separated in 2% agarose gels. Electrophoresis was performed in 19-cm-long gels made in 0.5×Tris-borate-EDTA buffer (Sigma-Aldrich, US) and run at 6 V/cm. In each run, the PCR product from the reference Mu50 (ATCC 700699) strain was included. The GeneRuler 100 bp DNA Ladder was utilized (Thermo Fisher Scientific, US). The gels were photographed under UV illumination. The size of the amplicons was measured with the assistance of the Uvitec-1D software v. 18.02. (Uvitec Cambridge, UK). The number of repeats was deduced using the Mu50 strain MLVA profile as a reference (Table 2) and subsequently employed to create MLVA profiles consisting of strings of 14 integers that were used for categorical clustering. A single change in the locus was considered a separate MLVA profile (genotype).

Table 2. VNTRs characteristic of the reference strain Mu50 [8]

VNTR locus	Size (bp) of repeat unit	No. of repeats	Amplicon size (bp)
Panel 1			
Sa0122	24	10	392
Sa0266	81	6	630
Sa0311	55	3	272
Sa0704	67	4	380
Sa1132	63	6	532
Sa1194	67	7	524
Sa1291	64	4	369
Sa1729	56	5	499
Sa1866	159	3	607
Sa2039	56	3	282
Panel 2			
Sa0906	56	3	864
Sa1213	56	5	868
Sa1425	58	4	630
Sa1756	131	2	365

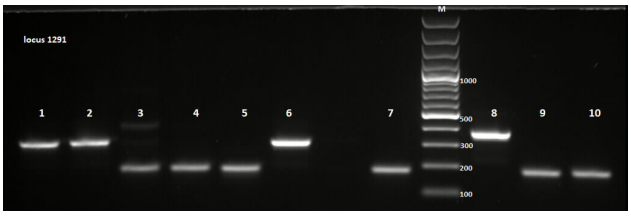
The MLVA profiles and allel size ranges have been deposited in the MLVA bank for Bacterial Genotyping (http://mlva.i2bc.paris-saclay.fr/mlvav4/genotyping/). Their dendrograms were constructed with the use of the ETE Toolkit software (http://etetoolkit.org/) based on the unique Newick code generated in the MLVA bank for the MLVA profiles of the SA isolates.

RESULTS

30 unique MLVA profiles (genotypes) were generated among the 31 SA isolates using Panel 1 (10 loci) of the applied MLVA scheme. Two isolates (no. 18 and 19) shared an identical MLVA pattern. The MLVA typing results, including sizes of the PCR products for the VNTRs (Panel 1) along with the calculated number of repeats, are demonstrated in Table 2. Polymorphism of selected VNTRs included in Panel 1 is shown by the agarose gel electrophoresis of amplicons in Figures 1 and 2.

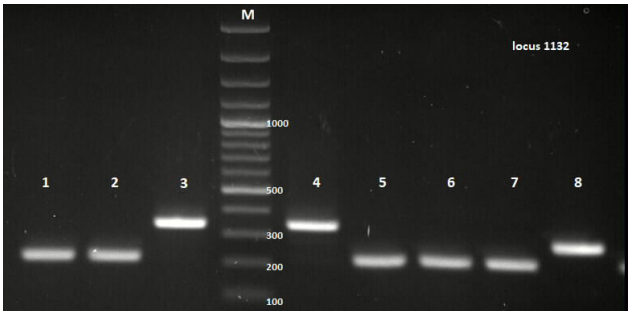
Three out of the four loci included in Panel 2 (Sa0906, Sa1213 and Sa1425) yielded small PCR products (below one repeat) for 18 (58%), which limited its discriminatory usefulness (Table 3). Examples of small amplicons for the selected loci are demonstrated in Figures 3 and 4. Due to the aforementioned atypical size of amplification products obtained in Panel 2, subsequent phylogenetic relationship between the isolates and their clustering were performed on the basis of the analysis of loci included in Panel 1 only.

In order to determine the principles of grouping MLVA patterns into clusters, various cut-off values regarding the similarity between MLVA patterns were tested. As an outcome, the cut-off value of ≥65% was used for further analysis. Two major genetic clusters (designated as I and II) were identified (Figure 5). Cluster I included 26 (84%) SA isolates, among which 14 were classified into five subclusters designated A-E. Cluster II included five SA isolates, among which two were grouped into sub-cluster F (isolates no. 22, 20). The remaining SA isolates from clusters I (isolates no. 47, 42, 03, 23, 17, 08, 38, 11, 16, 04, 41, 02) and II (isolates no. 21, 39, 07), respectively, represented unique MLVA patterns. The major sub-cluster B included five MLVA patterns represented by the corresponding number of isolates. Sub-clusters A, C, D and F were represented by two MLVA patterns each, and the corresponding number of isolates. Subcluster E included two MLVA patterns, among which one of the profiles was shared by two SA isolates (no. 18 and 19) cultured from patients hospitalized in the Intensive Care Unit. These clonal isolates were derived from blood and perinoneal fluid samples, respectively, and demonstrated an identical antibiotic resistance profile (inducible MLSB phenotype).



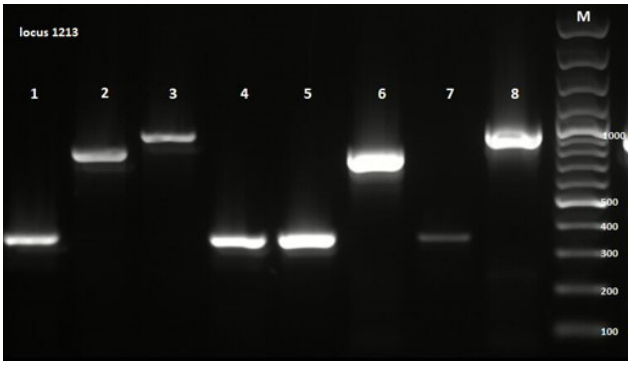
Lanes 1, 2, 6: 305 bp (repeat no.=3.0) amplicon; Lanes 3, 4, 5, 7, 9, 10: 177 bp (repeat no.=1.0) amplicon; Lane 8: 369 bp (repeat no.=4.0) amplicon (reference Mu50 strain); M – 100 bp DNA size marker

Figure 1. Polymorphism of Sa1291 (Panel 1) VNTR locus (lanes 1-10) shown by the agarose gel electrophoresis of the PCR products



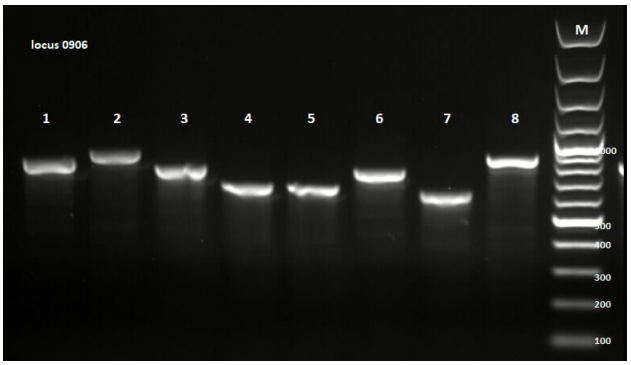
Lanes 1, 2, 5, 6, 7: 217 bp (repeat no.=1.0) amplicon; Lanes 3, 4: 343 bp (repeat no.=3.0) amplicon; Lane 8: 280 bp (repeat no.=2.0); M – 100 bp DNA size marker

Figure 2. Polymorphism of Sa1132 (Panel 1) VNTR locus (lanes 1-18) shown by the agarose gel electrophoresis of the PCR products



Lanes 1, 4, 5, 7: 364 bp (repeat no.<1.0; repeat no.=1.0 corresponds to the 644 bp PCR product) amplicon; Lanes 2, 6: 756 bp (repeat no.=3.0) amplicon; Lane 3: 868 bp (repeat no.=5.0) amplicon; Lane 8: 868 bp (repeat no.=5.0) amplicon (reference Mu50 strain); M – 100 bp DNA size marker

Figure 3. Polymorphism of Sa1213 (Panel 2) VNTR loci shown by the agarose gel electrophoresis of the PCR products



Lane 1: 864 bp (repeat no.=3.0) amplicon; Lane 8: 864 bp (repeat no.=3.0) amplicon (reference Mu50 strain); Lane 7: 640 bp (repeat no.<1.0; repeat no.=1.0 corresponds to the 752 bp PCR product) amplicon; Lanes 4, 5: 696 bp (repeat no.<1.0; repeat no.=1.0 corresponds to the 752 bp PCR product) amplicon; Lanes 3, 6: 808 bp (repeat no.=2.0) amplicon; Lane 2: 976 bp (repeat no.=5.0) amplicon; M – 100 bp DNA size marker

Figure 4. Polymorphism of Sa0906 (Panel 2) VNTR loci shown by the agarose gel electrophoresis of the PCR products

Table 3. MLVA results for the analysed isolates (Panel 1)

Isolate no. n=31	Amplification product size (bp) and tandem repeats number (1.0-10) at specific loci									
	Sa0122	Sa0266	Sa0311	Sa0704	Sa1132	Sa1866	Sa2039	Sa1194	Sa1291	Sa1729
01	296 (6.0)	711 (7.0)	162 (1.0)	447 (5.0)	217 (1.0)	607 (3.0)	282 (3.0)	457 (6.0)	305 (3.0)	499 (5.0)
02	248 (4.0)	670 (6.5)	272 (3.0)	380 (4.0)	532 (6.0)	607 (3.0)	282 (3.0)	390 (5.0)	369 (4.0)	499 (5.0)
03	392 (10.0)	792 (8.0)	162 (1.0)	246 (2.0)	343 (3.0)	607 (3.0)	226 (2.0)	524 (7.0)	305 (3.0)	387 (3.0)
04	272 (5.0)	711 (7.0)	327 (4.0)	514 (6.0)	532 (6.0)	607 (3.0)	226 (2.0)	725(10.0)	305 (3.0)	499 (5.0)
05	392 (10.0)	630 (6.0)	217 (2.0)	514 (6.0)	784 (10.0)	607 (3.0)	170 (1.0)	390 (5.0)	177 (1.0)	387(3.0)
06	392 (10.0)	630 (6.0)	217 (2.0)	514 (6.0)	658 (8.0)	607 (3.0)	170 (1.0)	390 (5.0)	177 (1.0)	387(3.0)
07	512 (15.0)	549 (5.0)	272 (3.0)	313 (3.0)	217 (1.0)	448 (2.0)	506 (7.0)	390 (5.0)	177 (1.0)	499 (5.0)
08	416 (11.0)	630 (6.0)	272 (3.0)	514 (6.0)	784 (10.0)	607 (3.0)	170 (1.0)	591 (8.0)	305 (3.0)	499 (5.0)
09	176 (1.0)	630 (6.0)	162 (1.0)	246 (2.0)	217 (1.0)	607 (3.0)	226 (2.0)	189 (2.0)	305 (3.0)	331 (2.0)
10	416 (11.0)	630 (6.0)	382 (5.0)	447 (5.0)	217 (1.0)	607 (3.0)	170 (1.0)	524 (7.0)	177 (1.0)	499 (5.0)
11	296 (6.0)	630 (6.0)	327 (4.0)	380 (4.0)	343 (3.0)	607 (3.0)	282 (3.0)	390 (5.0)	369 (4.0)	499 (5.0)
12	272 (5.0)	630 (6.0)	437 (6.0)	447 (5.0)	217 (1.0)	607 (3.0)	170 (1.0)	524 (7.0)	177 (1.0)	275 (1.0)
13	248 (4.0)	630 (6.0)	382 (5.0)	447 (5.0)	217 (1.0)	607 (3.0)	170 (1.0)	524 (7.0)	177 (1.0)	499 (5.0)
14	200 (2.0)	630 (6.0)	162 (1.0)	246 (2.0),	217 (1.0)	607 (3.0)	226 (2.0)	256 (3.0)	369 (4.0)	331 (2.0)
15	296 (6.0)	630 (6.0)	437 (6.0)	447 (5.0)	217 (1.0)	607 (3.0)	170 (1.0)	524 (7.0)	177 (1.0)	275 (1.0)
16	272 (5.0)	630 (6.0)	327 (4.0)	380 (4.0)	343 (3.0)	607 (3.0)	226 (2.0)	524 (7.0)	305 (3.0)	387 (3.0)
17	344 (8.0)	265 (1.5)	217 (2.0)	380 (4.0)	343 (3.0)	607 (3.0)	282 (3.0)	256 (3.0)	177 (1.0)	275 (1.0)
18	272 (5.0)	711 (7.0)	162 (1.0)	447 (5.0)	217 (1.0)	607 (3.0)	282 (3.0)	457 (6.0)	305 (3.0)	499 (5.0)
19	272 (5.0)	711 (7.0)	162 (1.0)	447 (5.0)	217 (1.0)	607 (3.0)	282 (3.0)	457 (6.0)	305 (3.0)	499 (5.0)
20	296 (6.0)	630 (6.0)	382 (5.0)	313 (3.0)	217 (1.0)	289 (1.0)	282 (3.0)	390 (5.0)	177 (1.0)	331 (2.0)
21	320 (7.0)	549 (5.0)	162 (1.0)	514 (6.0)	280 (2.0)	766 (4.0)	226 (2.0)	524 (7.0)	177 (1.0)	499 (5.0)
22	296 (6.0)	549 (5.0)	327 (4.0)	313 (3.0)	217 (1.0)	289 (1.0)	282 (3.0)	390 (5.0)	369 (4.0)	331 (2.0)
23	320 (7.0)	630 (6.0)	217 (2.0)	514 (6.0)	217 (1.0)	448 (2.0)	226 (2.0)	256 (3.0)	177 (1.0)	275 (1.0)
38	368 (9.0)	711 (7.0)	217 (2.0)	514 (6.0)	595 (7.0)	607 (3.0)	282 (3.0)	591 (8.0)	305 (3.0)	555 (6.0)
39	368 (9.0)	508 (4,5)	272 (3.0)	313 (3.0)	532 (6.0)	607 (3.0)	226 (2.0)	591 (8.0)	369 (4.0)	499 (5.0)
41	248 (4.0)	711 (7.0)	272 (3.0)	380 (4.0)	217 (1.0)	607 (3.0)	226 (2.0)	457 (6.0)	305 (3.0)	499 (5.0)
42	392 (10.0)	549 (5.0)	162 (1.0)	246 (2.0)	532 (6.0)	607 (3.0)	282 (3.0)	524 (7.0)	305 (3.0)	331 (2.0)
43	320 (7.0)	630 (6.0)	327 (4.0)	313 (3.0)	532 (6.0)	607 (3.0)	390 (5.0)	369 (4.0)	369 (4.0)	499 (5.0)
45	320 (7.0)	630 (6.0)	327 (4.0)	380 (4.0)	532 (6.0)	607 (3.0)	390 (5.0)	369 (4.0)	369 (4.0)	499 (5.0)
46	368 (9.0)	630 (6.0)	437 (6.0)	313 (3.0)	217 (1.0)	607 (3.0)	524 (7.0)	177 (1.0)	177 (1.0)	499 (5.0)
47	392 (10.0)	468 (4.0)	272 (3.0)	246 (2.0)	595 (7.0)	607 (3.0)	524 (7.0)	241 (2.0)	241 (2.0)	387 (3.0)

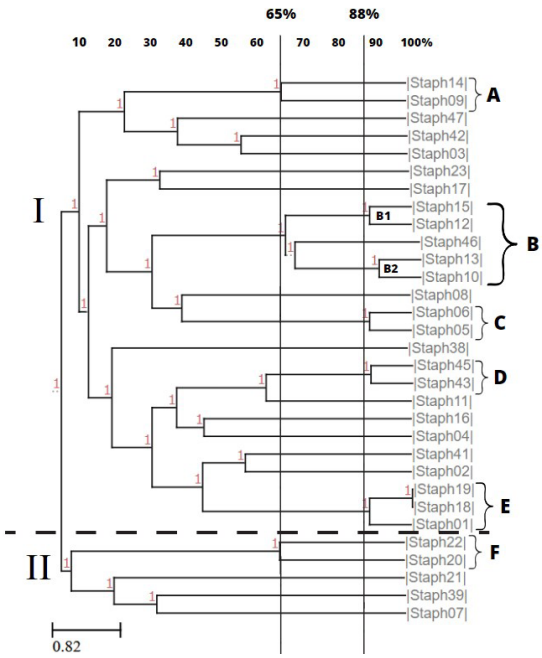


Figure 5. MLVA dendrogram of the study isolates; isolate clusters were delineated with the 65% and 88% similarity cutoff value

Isolates classified into sub-clusters B1 (isolates no. 12, 15), B2 (isolates no. 10, 13), C (isolates no. 05, 06), D (isolates no. 43, 45) and E (isolates no. 01, 18, 19) demonstrated genetic relatedness at the level of >88%. Isolates representing sub-clusters B1, C and E shared an identical antibiotic resistance profile, and were isolated from single hospital wards (Toxicology, Alergology and Intensive Care Units, respectively). None were MRSA (Table 1). Their MLVA profiles differed in the single VNTRs included in Panel 1 (Sa0122/spa gene for sub-clusters B1, B2 and E; Sa1132/SAV1078 gene for sub-cluster C) (Table 2). As mentioned above, several discrepant results were obtained for VNTRs included in Panel 2. Isolates included in the B1 sub-cluster differed in one band (696 bp and 640 bp PCR products) for the Sa0906 locus in Panel 2, making interpretation of the repeat number impossible. One repeat for this locus should have corresponded to the 752 bp amplicon size (Table 3). The B2 sub-cluster differed in two bands included in Panel 2 (locus Sa0906 [752 bp product corresponding to one repeat and 696 bp – too small to be interpreted] and locus Sa1756 [234 bp and 365 bp, corresponding to one and two repeats, respectively]). Moreover, the remaining two loci (Sa1213, Sa1425) in Panel 2 yielded PCR products of the same size (364 bp and 224 bp, respectively) for these isolates which, again, were too small to estimate the repeat number. One repeat for the Sa1213 and Sa1425 loci should have corresponded to the 644 bp and 456 bp products, respectively (Table 3). Isolates included in sub-cluster C differed in one locus (Sa1213) included in Panel 2 (1036 bp and 1092 bp products corresponding to 8 and 9 repeats, respectively) and showed the same small product (472 bp) for locus Sa0906, making the calculation of the repeat number impossible. The three isolates (no. 01, 18, and 19) representing sub-cluster E were cultured from patients hospitalized in the Intensive Care Unit and shared the same antibiotic resistance profile, as described previously. Isolate no. 01 differed from the two clonal isolates (no. 18, 19) in terms of a number of repeats in a single locus included in Panel 1 (Sa0122/spa gene [296 bp vs. 272 bp products corresponding to 6 and 5 repeats, respectively]) (Table 2) and in two loci (Sa0906 [752 bp vs. 808 bp products corresponding to 1 and 2 repeats, respectively] and Sa1756 [627 bp vs. 496 bp products corresponding to 4 and 3 repeats, respectively]) included in Panel 2 (Table 3). Also, all three isolates yielded a small, 364 bp product in the Sa1213 locus (Panel 2) for which the number of repeats could not be estimated.

Isolates representing sub-cluster D shared the same antibiotic resistance profile (MRSA and constitutive MLSB) but were recovered from two different wards (Gastrology and Internal Medicine Units) (Table 1). They differed in the number of repeats in a single locus (Sa0704/intergenic region [313 bp and 380 bp products corresponding to 3 and 4 repeats, respectively) included in Panel 1 (Table 3) and in two (Sa1213 [868 bp and 756 bp products corresponding to 5 and 3 repeats, respectively]) and Sa1425 [572 bp and 738 bp products corresponding to 3 and 6 repeats, respectively] loci included in Panel 2 (Table 4). As mentioned previously, amplification of small products for the three out of the four loci included in Panel 2 necessitated exclusion of this second-line panel from genetic clustering of the isolates.

DISCUSSION

Our study was focused on a short-time and local investigation of SA molecular epidemiology in the hospital setting. We report a high genetic diversity of the analysed isolates and suggest a limited degree of intra- and inter-ward SA transmission. Only one pair of SA isolates was identified clonal due to sharing an identical MLVA pattern. Nevertheless, it should be mentioned that a total number of 11 (35%) isolates were grouped into sub-clusters (B1-E) with a phylogenetic relatedness estimated at the level of >88% (based on the first-line Panel 1 clustering) which can suggest their related origin. Interestingly, the majority of SA isolates representing the above mentioned sub-clusters shared identical antibiotic resistance profiles and were recovered from the same hospital wards. Only one pair of isolates among those possibly related (sub-cluster C) included MRSA recovered from two different wards. Of note, only five (16%) MRSA isolates were identified during the study which correlates to a general low incidence of MRSA in the hospital.

The MLVA has been previously reported to represent a reliable typing method for short-term epidemiologic investigations of SA in the hospital setting [7,11,12] which supports its application in our 4-month study period. Several MLVA schemes have been developed for SA genotyping so far, including amplification of as many as 16 VNTRs in multiplex PCRs and a subsequent analysis of the products by capillary electrophoresis ensuring a high throughput and high discriminatory power [8,10,13-16]. Our study employed the 14 VNTR-based MLVA scheme developed by Pourcel et al. [8]. This approach is based on a two-step algorithm, including a first-line assay comprising a subset of 10 loci (Panel 1) and a second subset of 4 loci (Panel 2) that provides a higher resolution when required [8]. Pourcel et al. observed satisfying results with the analysis of 10 VNTRs included in

Table 4. MLVA results for the analysed isolates (Panel 2)

Isolate no. n=31	Amplification product size (bp) and tandem repeats number (1.0-9.0) at specific loci			
	Sa0906	Sa1213	Sa1425	Sa1756
01	752 (1.0)	364 (NE)	572 (3.0)	627 (4.0)
02	864 (3.0)	980 (7.0)	688 (5.0)	365 (2.0)
03	808 (2.0)	980 (7.0)	514 (2.0)	365 (2.0)
04	1032 (6.0)	980 (7.0)	630 (4.0)	889 (6.0)
05	472 (NE)	1036 (8.0)	514 (2.0)	365 (2.0)
06	472 (NE)	1092 (9.0)	514 (2.0)	365 (2.0)
07	808 (2.0)	364 (NE)	224 (NE)	627 (4.0)
08	1088 (7.0)	1036 (8.0)	630 (4.0)	889 (6.0)
09	752 (1.0)	644 (1.0)	572 (3.0)	889 (6.0)
10	752 (1.0)	364 (NE)	224 (NE)	234 (1.0)
11	1032 (6.0)	868 (5.0)	688 (5.0)	365 (2.0)
12	696 (NE)	364 (NE)	224 (NE)	365 (2.0)
13	696 (NE)	364 (NE)	224 (NE)	365 (2.0)
14	752 (1.0)	644 (1.0)	572 (3.0)	889 (6.0)
15	640 (NE)	364 (NE)	224 (NE)	365 (2.0)
16	640 (NE)	812 (4.0)	630 (4.0)	365 (2.0)
17	864 (3.0)	756 (3.0)	514 (2.0)	365 (2.0)
18	808 (2.0)	364 (NE)	572 (3.0)	496 (3.0)
19	808 (2.0)	364 (NE)	572 (3.0)	496 (3.0)
20	752 (1.0)	756 (3.0)	224 (NE)	496 (3.0)
21	528 (NE)	812 (4.0)	514 (2.0)	365 (2.0)
22	752 (1.0)	756 (3.0)	224 (NE)	496 (3.0)
23	808 (2.0)	644 (1.0)	572 (3.0)	365 (2.0)
38	1032 (6.0)	924 (6.0)	630 (4.0)	496 (3.0)
39	1032 (6.0)	868 (5.0)	688 (5.0)	365 (2.0)
41	864 (3.0)	364 (NE)	688 (5.0)	496 (3.0)
42	528 (NE)	364 (NE)	514 (2.0)	234 (1.0)
43	1032 (6.0)	868 (5.0)	572 (3.0)	365 (2.0)
45	1032 (6.0)	756 (3.0)	738 (6.0)	365 (2.0)
46	752 (1.0)	364 (NE)	224 (NE)	365 (2.0)
47	584 (NE)	924 (6.0)	630 (4.0)	496 (3.0)

NE – not estimated

Panel 1 and recommended its use for basic clustering. Loci included in Panel 2 yielded very small alleles in certain cases in their study, therefore, its application was recommended as a second-line and a more discriminatory approach. Unexpectedly, but in line with this publication, the MLVA assay applied in our study yielded small amplicons for as many as three out of the four loci included in Panel 2 for more than half of the isolates. The small sizes of the amplicons made the calculation of the number or repeats impossible, therefore, the resolution capability of Panel 2 was significantly limited in our research. Nevertheless, since the majority of SA isolates demonstrated a high degree of genetic diversity at the first step of the VNTR analysis, we continued subsequent clustering using results obtained with the Panel 1. Interestingly, the clonal pair of isolates (no. 18 and 19) identified by the Panel 1 analysis, yielded an identical MLVA pattern in Panel 2 as well, including a small amplification product in the locus Sa1213 for which the number of repeats could not be calculated [8].

Isolates grouped into sub-clusters B1-E with an established ≥88% similarity in Panel 1, yielded different sizes (including those with a size below a single repeat) of the PCR products for up to two loci in Panel 2. It is, therefore, probable that the polymorphism of the Panel 2 VNTR loci could decrease the overall degree of genetic similarity within these sub-clusters and reveal even greater diversity within the studied isolates. The three loci included in Panel 2 (Sa0906, Sa1213, and Sa1425) for which the small PCR products were obtained correspond to repeat (STAR) elements representing a family of intergenic elements found in many copies throughout the SA genome. Their sequence was reported to consist of several small and unusually GC-rich direct repeats with recurring intervening sequences [18]. It is conceivable that these highly complex and polymorphic loci could have undergone additional genetic rearrangements, including deletions, giving rise to the small amplicons. To support this hypothesis, however, further studies based upon sequencing will be necessary. Sequencing of the whole genome will be most informative since the sequence analysis of the small PCR product may not reveal the actual genetic events standing behind this anomaly.

The major constraint of this study is a small number of SA isolates subjected to the molecular investigation. Nevertheless, we managed to genotype all clinical SA isolates that were cultured in the hospital laboratory during a 4-month period. Their low prevalence, as well as a low incidence of infections caused by MRSA in the hospital does not eliminate a necessity to detect and control the spread of this microorganism. Our study indicates the usefulness of the MLVA tool comprising a primary subset of 10 loci to track the possibility of SA intra-hospital transmission. Further studies are necessary to explain the anomaly associated with small alleles obtained for the Panel 2 loci, as their subset can provide even higher resolution of the assay.

CONCLUSIONS

The MLVA method presented in this study is a simple, efficient and reliable method of SA genotyping that allows determining the relatedness of strains and their clonality, and thus facilitates the control of their spread in the hospital environment. This method, by demonstrating the genetic heterogeneity of the VNTR loci among isolates, ensures quick and reliable generation of genotype data, and is also a good tool for hospital molecular epidemiology surveillance, which, in turn, leads to a comprehensive insight into the population genetics and evolution of this microorganism.

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