Bacterial fingerprints across Europe

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CHAPTER 2

Rapid and high-resolution distinction of community-acquired and nosocomial *Staphylococcus aureus* isolates with identical pulsed-field gel electrophoresis patterns and *spa*-types

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) represents a serious threat for public health worldwide. Of particular concern is the emergence of community-acquired MRSA, which is often difficult to distinguish from nosocomial MRSA due to a lack of suitable typing methods for early detection. For example, the USA300 pulsed-field gel electrophoresis (PFGE) pattern includes both the classical community-acquired USA300 clone with *spa*-type t008 and an epidemiologically unrelated nosocomial clone with *spa*-type t024. Likewise, *spa*-typing cannot distinguish the classical USA300 from nosocomial MRSA with the *spa*-type t008. Since the fast and high-resolution distinction of these *S. aureus* types is important for infection prevention and surveillance, we investigated whether multiple-locus variable number tandem repeat (VNTR) fingerprinting (MLVF) can be applied to overcome these limitations. Indeed, MLVF correctly grouped 91 MRSA isolates belonging to the classical USA300 lineage, nosocomial MRSA isolates with the USA300 PFGE profile and *spa*-type t024, and nosocomial MRSA isolates with *spa*-type t008 into three distinct clusters. Importantly, several sub-clusters were also identified, reflecting epidemiological relationships between the respective isolates. We conclude that MLVF has the discriminatory power needed to rapidly distinguish very similar community-acquired and nosocomial MRSA isolates, and that MLVF-based sub-clustering of isolates is highly useful for epidemiological investigations, outbreak prevention and control.

INTRODUCTION

The epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) has changed drastically over the past decade especially due to the emergence of highly transmissible community-acquired lineages. MRSA infections were originally a nosocomial challenge, but now they have also expanded to the community and livestock [1-4]. Consequently, infectious diseases caused by this Gram-positive pathogen have become an even more serious threat for public health worldwide. One alarming and rapidly spreading MRSA is the type USA300, which was first reported in the United States of America in 2001 and disseminated throughout the community and subsequently to hospitals across all continents within a decade [1-8]. A major problem here is that the community-acquired MRSA is often difficult to distinguish from nosocomial MRSA due to a lack of suitable typing methods for early detection.

Molecular typing of clinical *S. aureus* isolates is important to decide on effective surveillance and control strategies as well as to understand the rapid spread, the complex population biology and the infectious status of this rapidly evolving pathogen. To monitor the emergence of new *S. aureus* strains, various typing techniques have been developed, including pulsed-field gel electrophoresis (PFGE), multiple-locus variable number tandem repeat (VNTR) analysis/fingerprinting (MLVA/MLVF) and *spa*-typing. The latter has become a highly popular and widely utilized typing approach, in particular for surveillance and broad epidemiological studies at the international level. For *spa*-typing, the protein A (*spa*) gene is PCR-amplified and sequenced. Based on the detected VNTRs and their composition, a specific *spa*-type is assigned to the investigated *S. aureus* isolate [9,10]. The key advantages of this approach are the production of transportable data and the good accessibility of the RIDOM *spa* server. Additionally, *spa*-typing can be performed in a high-throughput manner, is highly reproducible and cheap. However, *spa*-typing is limited by the fact that it is a single-locus typing approach that is less discriminatory than PFGE [11,12], which is in many countries still regarded as the 'gold standard' in typing of *S. aureus* for infection control and outbreak analysis.

Although it has a high discriminatory power, PFGE is relatively labour-intensive and expensive. Moreover, the inter-laboratory comparison of data produced by PFGE is challenging [13]. Importantly, several studies have recently shown that related strains with indistinguishable PFGE profiles can be further differentiated with PCR-based typing methods [1-4,14,15]. Specifically, Larsen *et al.* showed that PFGE was unable to distinguish the 'classical' USA300 clone with the *spa*-type t008 from a newly described clone with the *spa*-type t024, both belonging to the multiple locus sequence type 8 (ST8) lineage [1-6,8,15,16]. This implies that the identification of USA300 isolates is often imprecise since it was so far mainly based on the PFGE nomenclature. The high similarity between these two clones is underscored by the fact that their *spa* genes differ only in one VNTR. Furthermore, other molecular markers are associated with both clones, such as the staphylococcal cassette chromosome *mec* (SCC*mec*) types IV or V. On the other hand, it was shown that these isolates differed in several other molecular markers, such as the type of direct repeat units (*dru*), the arginine catabolic mobile element (ACME), and the Panton-Valentine leukocidin (PVL) genes *lukS-PV* and *lukF-PV* [9,10,17-20]. One crucial difference between these two clones is that the classical USA300 clone is community-acquired, whereas isolates with the USA300 PFGE profile and *spa*-type t024 were solely encountered in hospitals and nursing homes [11,12,15].

Intriguingly, *spa*-typing cannot distinguish the classical USA300 isolates from MRSA isolates of the hospital-acquired PVL-negative ST8 lineage [13,21], as both have the *spa*-type t008. Thus, both PFGE and *spa*-typing have difficulties in distinguishing the classical USA300 clone from other clones with different genotypes and epidemiological backgrounds. Whereas Denmark is struggling more with the distinction of classical USA300 isolates from nosocomial MRSA isolates with the same PFGE profile, the challenge for outbreak prevention and detection in other parts of Europe is to distinguish hospital-acquired isolates with the *spa*-type t008 from the classical USA300 isolates [21].

MLVF is an attractive high-throughput alternative for PFGE that is complementary to *spa*-typing [22]. This approach is based on PCR amplification of five staphylococcal VNTR loci (*sdr, clfA*, *clfB*, *sspA*

and *spa*). The number of repeated units at the same locus, which vary from isolate to isolate, can be detected by PCR with flanking primers and this parameter is therefore indicative for the relationships between isolates. Moreover, as shown by Sabat *et al*., a recently updated version of the MLVF protocol employing a new set of primers and microcapillary electrophoresis for the separation of amplified fragments is highly suitable for the identification of *S. aureus* outbreaks and strain transmission events [8].

The present studies were aimed at determining whether MLVF can overcome the limitations of *spa*-typing and PFGE in distinguishing highly similar community-acquired and nosocomial *S. aureus* isolates. To this end, we performed an MLVF analysis on isolates of the classical USA300 clone, isolates with the USA300 PFGE profile and *spa*-type t024, and isolates of the hospital-acquired ST8 lineage (Lyon clone) with the related *spa*-types t008, t024, t068 and t530. Indeed, MLVF was capable of rapidly and precisely distinguishing these three groups of isolates and its high resolution allowed sub-clustering of isolates with related epidemiology. This highlights MLVF as a useful tool for surveillance and infection control.

MATERIALS AND METHODS

Bacterial isolates

A total of 91 MRSA isolates were investigated in the current study. All isolates are listed in the Supplementary Table 1 in accordance with their clustering by MLVF, and with relevant molecular and epidemiological information. The 78 *S. aureus* isolates with the USA300 PFGE profile were collected at the Statens Serum Institut (Copenhagen, Denmark) between 1999 and 2006 [15,23]. These *S. aureus* isolates were extensively characterized at the molecular and phenotypic level and in addition all clinical and epidemiological information was compiled [23]. Furthermore, two control isolates with the unrelated *spa*-types t002 and t037 were derived from the collection at the Statens Serum Institut. The remaining 11 isolates were selected from a collection of about 1,500 *S. aureus* isolates obtained from patients hospitalized between 1996 and 2010 in hospitals located within the Dutch-German border area (EUREGIO). All of these isolates were characterized by *spa*-typing and microarray analyses (Alere Technologies, Jena, Germany), allowing their assignment to distinct lineages [21]. The 11 isolates included in the present study belong to the *spa*-CC008 lineage, have the SCC*mec* type IV and are PVL-negative. Notably, all 11 isolates belong to the so-called Lyon clone (also named UK-EMRSA-2) and include 8 isolates with the *spa*-type t008, and individual isolates with the related *spa*-types t024, t068 and t530.

For control purposes, the *S. aureus* clinical MRSA isolate M2, isolated at the University Medical Center Groningen (UMCG, Groningen, the Netherlands), was included in all performed experiments [8]. Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2012.12.005.

Bacterial growth and genomic DNA extraction

All isolates were stored at -80 °C. Total DNA was prepared from five to ten colonies lifted from blood agar plates incubated for 24 h at 37 °C and suspended in 500 µL of Tris-EDTA (TE) buffer (pH 8.0). The cell suspension was transferred to 2 ml bead-beating tubes with screw caps containing zirconia/silica beads with a diameter of 100 µm. The tubes were fixed in a Precellys bead beater, which was operated 3 times for 30 s per pulse at a speed of 5,000 rpm, with 30 s intervals between pulses. Subsequently, the samples were heated for 10 min at 95°C and centrifuged (14,000 rpm) for 10 min at 4°C. Finally, 200 µL of the suspension was transferred to a fresh tube and stored at -20°C until further use. For microarray analysis, an inoculation loop of bacteria was homogenized with a **TissueLyser** II (Qiagen, Hilden, Germany), and genomic **DNA** was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

Multiple-locus variable number tandem repeat fingerprinting (MLVF)

MLVF was performed according to the improved protocol described by Sabat *et al.* [8]. Briefly, 1 µL of genomic DNA was subjected to a multiplex PCR in which seven staphylococcal VNTRgenes (*sdrC, sdrD, sdrE, clfA, clfB, sspA* and *spa*) are amplified. Subsequently, the PCR products were separated using the 2100 Bioanalyzer with microfluidic DNA 7500 Chips (Agilent Technologies, Palo Alto, USA), following the manufacturer's instruction. Each Bioanalyzer run included a control PCR product of the clinical isolate M2 to ensure the reproducibility of the data generated with the Bioanalyzer.

Data analysis of the MLVF results was carried out using the GelCompar II software from Applied Maths (Kortrijk, Belgium). MLVF patterns created by the 2100 Bioanalyzer software were imported as CSV files into the GelCompar II software. The position tolerance and optimization were set to 0.6% and 0.5%, respectively, and the dice formula was used to calculate the pairwise similarity coefficient. With the selected position tolerance, all Bioanalyzer runs for the control isolate M2 were identical. A dendrogram was created with the unweighted pair-group method using geometric averages (UPGMA). For distinguishing clusters the cut-off value was set at 67% [8]. Only when two patterns were identical they were regarded as the same subtype. MLVF patterns differing by one or more bands were considered as distinct patterns.

spa-typing

spa-typing was performed according to Harmsen *et al.* using the *spa* server [9,24].

DNA microarray typing

The StaphyType kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis. The StaphyType kit consists of DNA probes printed onto an array for detection of 334 *S. aureus* gene sequences, including species-specific, antimicrobial resistance and virulence genes as well as typing markers. The DNA microarray protocol was performed according to the manufacturer's instructions.

RESULTS

DNA fingerprinting with MLVF

MLVF grouped 89 isolates into three main clusters denoted as B (n = 11), C (n = 38) and E (n = 40) (Figure 1 and Supplementary Table 1). Two control isolates with the *spa*-types t002 and t037 had separate positions in the dendrogram (denoted as 'sporadic A' and 'sporadic D', respectively). Importantly, cluster B was exclusively composed of the eleven hospital-acquired MRSA isolates belonging to the Lyon clone, cluster C of the 38 classical USA300 isolates, and cluster E of the 40 isolates with USA300 PFGE profile and *spa*-type t024. As shown in Figure 1, the clusters B, C and E were composed of several different banding patterns. Cluster B featured six different patterns, cluster C ten and cluster E five. The two major sub-clusters C1 and E3 contained 23 and 30 isolates, respectively, each sub-cluster displaying exactly the same MLVF pattern. Thus, the majority of the isolates within each of the three main clusters are genetically very closely related but, more importantly, MLVF is able to define specific sub-clusters. Furthermore, the difference in the MLVF banding patterns can be related to differences in the respective *spa* genes (Figure 1, second band from the right), reflecting the *spa*-types t008 and t024 in clusters C and E, respectively. In particular, all isolates of cluster C had the *spa*-type t008, whereas all isolates of cluster E had the *spa*-type t024. In addition, the two distinct clusters also differed in the numbers of VNTRs in the bands representing *clfA*, *clfB* and the *sdr* locus (bands on the left in Figure 1). Only the VNTR numbers in the *sspA* locus were the same in all isolates of clusters C and E. It is noteworthy, that all isolates in clusters C and E contain all seven genes targeted by MLVF, which is reflected by the seven respective bands that were electrophoretically separated. In cluster E, the upper band (Figure 1, left) is in fact a double band relating to the *clfA* and *clfB* genes. This was independently confirmed by simplex PCR reactions for both genes in a number of representative isolates (data not shown).

The MLVF cluster B, which was solely composed of the 11 hospital-acquired MRSA isolates recovered from the EUREGIO was found to be relatively heterogeneous. This cluster featured 6 sub-clusters including 1, 2 or 5 isolates (Figure 1). Interestingly, in 8 of these isolates only 5 out of 7 possible bands were amplified, which probably relates to an incomplete *sdr* locus [25]. Furthermore, three isolates in cluster B (HA-E944, HA-E431 and HA-E276) have distinctive *spa*-types that grouped them in individual sub-clusters.

Epidemiological relevance of the MLVF clustering

In order to assess the epidemiological relevance of the MLVF sub-clustering in relation to *spa*-typing, the clinical data available for the isolates in each sub-cluster were compared (Supplementary Table 1). This revealed several cases of very likely transmissions: two isolates of sub-cluster C1 were derived from patients who lived together; the sub-cluster E1 consisted of three isolates which were derived from patients staying at the same hospital within a half-year period; the sub-cluster C4 included three isolates of which two came from patients who lived together, and the same is true for the two isolates in sub-cluster C6. Furthermore, 10 out of the 30 isolates in the large sub-cluster E3 were obtained from patients living in two nursing homes located in the same area of Copenhagen, Denmark. Moreover, 18 other isolates from sub-cluster E3 also originated from this particular area in Copenhagen. On the other hand, 5 isolates from this area grouped into different sub-clusters. Altogether, we conclude that MLVF was able to uncover epidemiological connections between the investigated isolates. This underpins the view that MLVF is exquisitely suited to trace back chains of staphylococcal transmission especially when combined with *spa*-typing.

DISCUSSION

In the present study, the recently improved MLVF protocol for *S. aureus* typing to distinguish highly similar community- and hospital-acquired MRSA isolates was applied [8]. The results show that MLVF has the required discriminatory power to differentiate between the classical USA300 isolates, isolates with USA300 PFGE profile and *spa*-type t024, and hospital-acquired MRSA isolates with the related *spa*-types t008, t024, t068 and t530. Additionally, MLVF allows a more detailed differentiation of isolates with the same *spa*-type into sub-clusters. To our knowledge, this is the first time that such a high resolution of related *S. aureus* clones has been achieved with a simple PCR-based typing tool [26]. This underscores the applicability of MLVF for surveillance, outbreak prevention and infection control.

Several studies have shown in recent years that the discriminatory power of MLVF is comparable to that of PFGE, but higher than that of MLVA, *spa*-typing and other PCR-based methods [8,11,12,26,27]. The first report on the limitations of PFGE in the differentiation of USA300 isolates was published in 2008 by the group of Patel *et al.* [28]. Furthermore, a study by Moser *et al.* showed that MLVF (Note that in earlier studies by Moser *et al.* (2009) and Tenover *et al.* (2007) MLVF was denoted as MLVA) was able to discriminate clinical isolates with common PFGE types and allowed the identification of unrelated and closely related isolates with identical PFGE patterns [29]. On the other hand Tenover *et al.* used a diverse collection of methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA isolates representing known USA types to demonstrate that MLVF could discriminate between isolates with the same PFGE profile [14]. Similarly, Karynski *et al.* showed that the sub-clustering of a large and diverse collection of clinical isolates with the same *spa*-type could be achieved by MLVF [30]. In a more recent publication, Holmes *et al.* applied MLVF to distinguish the epidemic and highly clonal lineages EMRSA-15 and EMRSA-16, which display in most cases indistinguishable PFGE profiles [27]. In the latter study, MLVF was shown to be more discriminative than MLVA and PFGE, and it was concluded that MLVF is an attractive typing tool for the investigation of hospital outbreaks. This view is fully supported by our recent studies in which MLVF was applied to distinguish 206 non-repetitive MRSA isolates recovered from infected patients at the UMCG between 2000 and 2010 [8].

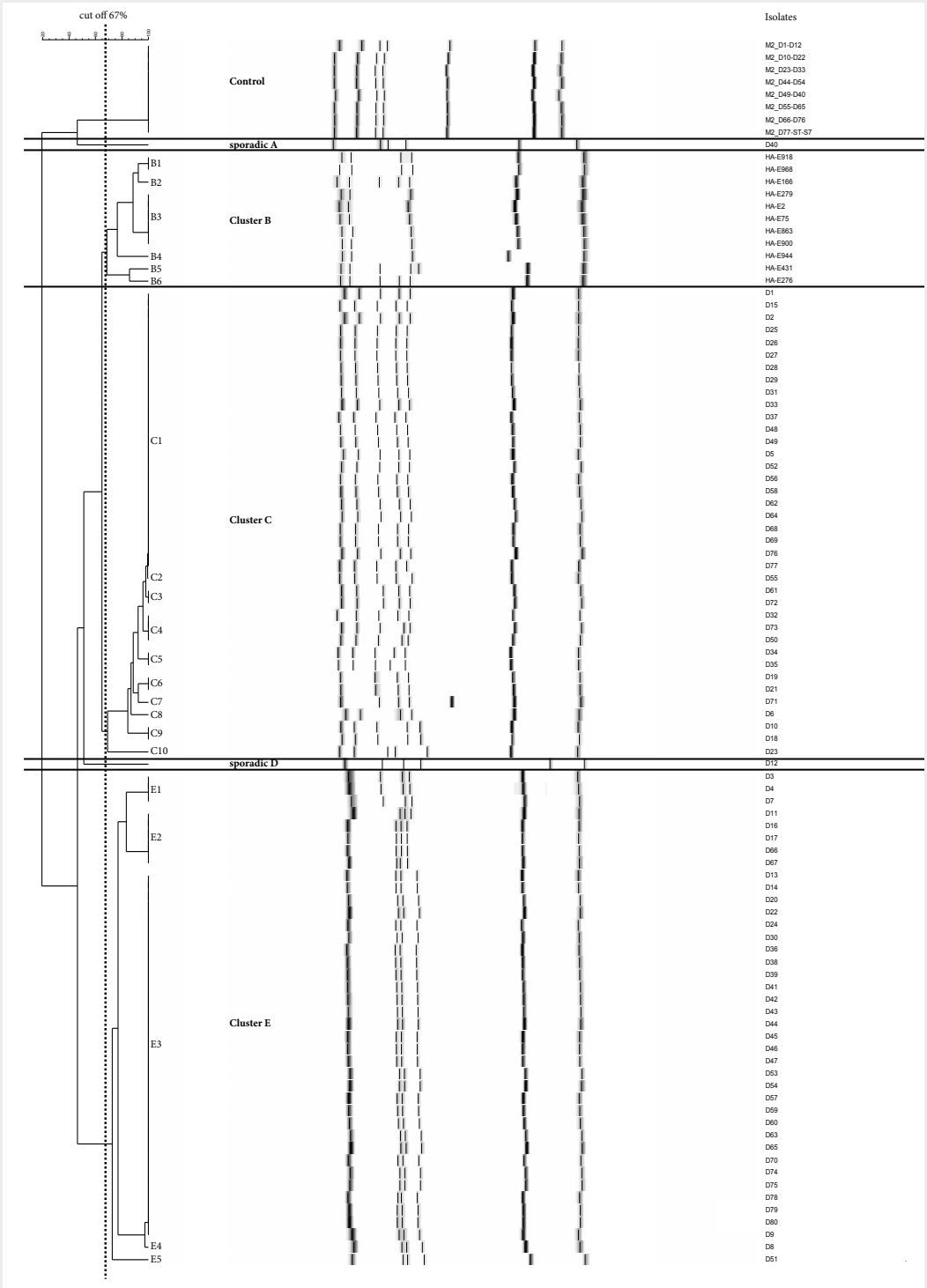

Figure 1. MLVF dendrogram of the 91 *S. aureus* isolates generated by the UPGMA algorithm. Clusters of the isolates were generated with a cut-off value set to 67%. Additionally to the 89 study isolates, 2 control isolates with different *spa*-types and 9 control samples (M2) were included in this delineation. The names of the isolates and clusters are indicated in the dendrogram.

In all former studies on the typing of *S. aureus* by MLVF, this technique was consistently recommended as a good and rapid tool for identifying outbreaks and for studying nosocomial MRSA infections in healthcare settings. In these studies the major goal was solely to determine the relatedness and epidemiology of isolates that had been collected over relatively short periods of time. Additionally, the central message in all these studies was that MLVF can replace the standard typing methods in local laboratories as a high-throughput surveillance screening tool for the identification and discrimination of clinical isolates. Its discriminative power for molecular typing was comprehensively demonstrated and, in addition, MLVF was reported to be fast, cheap and easy to apply. While the non-transportability of the produced data is a potential drawback, this is not a serious problem for applications at the local level.

Recent studies have shown a great spatial and temporal genetic variability in USA300 isolates from Europe and the USA [31,32]. Although the identified genetic changes were relatively small, they can have a significant impact on the overall virulence potential, resistance to antibiotics and interactions with the host. Accordingly, there is a clear need for highly discriminatory typing tools to distinguish individual representatives of this clone. As shown in the present study, these requirements are met by MLVF. Clearly, MLVF successfully distinguished the classical USA300 clone, which is PVL-positive and has the *spa*-type t008, from the t024 PVL-negative clone with a PFGE pattern indistinguishable from that of the USA300 clone. This is important because these clones also have very different epidemiological characteristics. The classical clone is typically community-acquired and encountered in patients with an average age of 31 years. In contrast, the isolates with the USA300 PFGE profile and *spa*-type t024 identified by Larsen *et al.* are mainly nosocomial or associated to nursing homes and encountered in patients with an average age of 72 [15]. This difference in epidemic behaviour may relate to the loss of the PVL genes from all and loss of ACME in 80% of the isolates with the USA300 PFGE profile and *spa*-type t024. ACME is a hallmark of the classical USA300 clone that has been implicated in its virulence and fitness [17,18,33]. The frequent absence of ACME in the t024 isolates as well as the loss of the PVL gene and one repeat within the VNTR in the *spa* gene suggest that the t024 isolates have evolved from the classical USA300 clone, but this idea still needs to be confirmed by genome sequencing since it is also possible that these clones have evolved independently. Importantly, the distinct clustering of the 11 hospital-acquired PVL-negative MRSA isolates with *spa*-type t008 or closely related *spa*-types singles out MLVF as a particularly useful tool for identifying *S. aureus* isolates that are indistinguishable by other typing approaches.

Due to its very high discriminatory power, ease-of-use and low costs, MLVF is a very attractive typing tool for (i) the prevention and detection of MRSA outbreaks, (ii) the identification of sources and routes of transmission and (iii) the identification of closely related *S. aureus* isolates for epidemiological and evolutionary studies, especially in local healthcare settings. The present studies show that MLVF has a higher discriminatory power when applied to highly similar *S. aureus* clinical isolates than PFGE and *spa*-typing, and that the MLVF sub-clustering is meaningful in terms of epidemiology. Further characterization of these sub-clusters, for example by high-throughput proteomics, will ultimately lead to a better understanding of critical determinants for fitness in community-acquired and nosocomial lineages of *S. aureus*.

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‘Träume nicht dein Leben, sonder lebe deinen Traum.’