Chapter 15

spa Typing for Epidemiological Surveillance of *Staphylococcus aureus*

Marie Hallin, Alexander W. Friedrich, and Marc J. Struelens

Abstract

The *spa* typing method is based on sequencing of the polymorphic X region of the protein A gene (*spa*), present in all strains of *Staphylococcus aureus*. The X region is constituted of a variable number of 24-bp repeats flanked by well-conserved regions. This single-locus sequence-based typing method combines a number of technical advantages, such as rapidity, reproducibility, and portability. Moreover, due to its repeat structure, the *spa* locus simultaneously indexes micro- and macrovariations, enabling the use of *spa* typing in both local and global epidemiological studies. These studies are facilitated by the establishment of standardized *spa* type nomenclature and Internet shared databases.

Key words: Epidemiology; methicillin resistance; phylogeny; *S. aureus*; sequence analysis; software; staphylococcal protein A; typing methods.

1. Introduction

1.1. Typing Methods Available for Staphylococcus aureus

Staphylococcus aureus is a leading human pathogen responsible for a wide range of diseases, from superficial skin infections to life-threatening conditions, such as bacteremia, endocarditis, pneumonia, or toxic shock syndrome (*1*). Since the early 1960s, when they first emerged (*2*), strains of *S. aureus* resistant to methicillin and other β-lactams (MRSA) have spread worldwide and caused outbreaks in the hospital setting as well as in the community, thereby becoming a major public health threat (*3*). During the last decades, diverse typing methods, first phenotypic, then genotypic,

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have been used for monitoring *S. aureus* spread. Among these, pulsed-field gel electrophoresis (PFGE) of genomic macrorestriction fragments is considered the gold-standard method (***4***). However, PFGE is a technically demanding and labor-intensive method. Moreover, its interpretation leaves room to subjectivity (***5***), and interlaboratory results comparison remains difficult and subject to strict adherence to standardized protocols and interpretation criteria (***6–8***).

Multilocus sequence typing (MLST), based on the sequence polymorphism of approx 500-bp long fragments of seven housekeeping genes was designed to study the *S. aureus* population genetic structure. This technique, applied to large *S. aureus* strain collections, revealed that the population structure is essentially clonal, and that the large majority of epidemic MRSA clones belong to a few phylogenetically distinct lineages or clonal complexes (CCs) (***9***).

MLST has also proved to be adequate for long-term global epidemiology and the study of recent evolution of *S. aureus* (***9,10***). However, MLST typing remains too expensive and labor intensive for its application to outbreak investigations and routine surveillance (***10,11***).

In recent years, more focused sequence-based methods have been developed to provide fast, unambiguous, and exportable typing data. Among these, the sequence determination of the polymorphic X region of *spa* gene, called *spa* typing, is gaining favor as a reliable tool for typing *S. aureus*. Frenay et al. were, in 1994, the first team to target the polymorphic X region of *spa* gene as an epidemiological marker. At that time, the X region was amplified and its size estimated by electrophoresis (***12***). In 1996, the same team improved the technique by performing sequence analysis of the X region (***13***). Since then, many studies have evaluated the usefulness of this technique for diverse epidemiological purposes and confirmed its ease of use and speed. Initially, two limitations hampered use of *spa* typing for surveillance: the lack of software capable of identifying and clustering repeat units and profiles and the lack of consensus nomenclature allowing interlaboratory exchange of results. These limits have been recently overcome, making *spa* typing a prime alternative to PFGE for typing *S. aureus*. In this chapter, we outline the biological basis of *spa* locus polymorphism and performance of *spa* sequence typing and describe the methods of analysis and data interpretation as well as international *spa* typing networks.

1.2. Structural Specificity of the spa Gene Hypervariable X Region

Protein A is a cell-wall component bound to the peptidoglycan of *S. aureus* by its COOH-terminal part. It interacts with the Fc-fragment of immunoglobulins by its NH_2-terminal part and thereby inhibits phagocytosis by polymorphonuclear leucocytes. The *spa* gene is composed of an N-terminal region encoding four

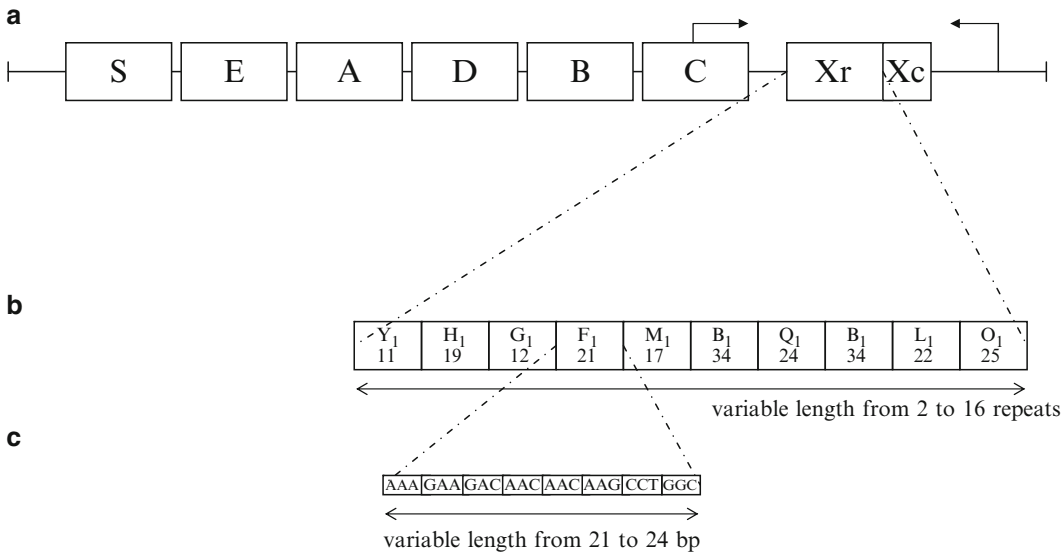

Fig. 1. (**a**) Schematic map of the *spa* gene. (Adapted from refs. *18, 20, 41*.) S is a signal sequence; A to D are IgG-binding domains; X is the C-terminal part, divided in two regions, the VNTR region (Xr) and a constant region coding for cell wall attachment (Xc). Arrows indicate the primers' localization. (**b**) The repeat structure of the Xr region. The *spa* type illustrated is t008 (Ridom-Harmsen et al. nomenclature) or YHGFMBQBLO (Kreiswirth nomenclature). (**c**) The DNA sequence of the *spa* repeat 21 (Ridom) or -F_1 (Kreiswrirth) repeat.

to five homologous immunoglobulin G (IgG) binding units, while its C-terminal sequence, called the X region, exhibits a variable number of short (24-bp) repeated units flanked by well-conserved regions (**Fig. 1**) (*14*).

The origin of the so-called variable number of tandem repeats (VNTR) structure of the X region is explained by the slipped strand mispairing model. Basically, illegitimate basepairing due to stretches and loops in short repeated unit motifs occurs during DNA replication, leading the DNA polymerase to delete or insert repeat units (*15*).

This VNTR structure is specific to neither the *spa* gene nor *S. aureus*. These hypervariable regions have been identified in many bacterial species and are frequently used as epidemiological markers. For example, the *spa* gene is one of the targets used by several multilocus VNTR analysis (MLVA) typing systems developed for *S. aureus*, together with *sdrC, D*, and *E, sspA, ClfA* and *B*, *cna*, or *fnBP* genes (*16*).

1.3. spa Typing Nomenclature

Two *spa* type designations have been used in the last years, one developed by Harmsen et al. (*17*) and one developed by Kreiswirth et al. (*18*). The latter has been changed recently, and comparison between the old designation and the new designation as well as a comparison between the Harmsen and the Kreiswirth nomenclature is only possible via computerized tools. The general

approach is, however, similar for both nomenclatures: Each repeat identified is associated to a code (numerical for the Harmsen et al. and alphanumerical for the Kreiswirth et al. nomenclature). Each *n*-long repeat profile corresponds to an *n*-long code constituted by the succession of the repeat's codes, as illustrated in **Fig. 1B**. In addition, in the Harmsen et al. nomenclature, a "type" number preceded by the letter t is then assigned to every distinct repeat profile (*see* **Note 1**). Due to its broader international use, we focus in the following section on the Harmsen et al. (Ridom) spa type designation.

1.4. spa *Typing Performance and Application Field*

As a sequence-based method, *spa* typing possesses many obvious advantages, such as rapidity, ease of use, suitability for computerized analysis, storage, and (ex)portability of results (***19***).

1.4.1. Typeability, Reproducibility, and Stability

Typeability is virtually 100%, although mutations in the flanking conserved regions of the X region, used for primer design, have occasionally been described (***20***), leading to amplification problems. However, a limitation of the clustering analysis may occasionally occur with isolates presenting short repeat profiles. Indeed, the epidemiological information contained by these profiles may be insufficient to permit reliable clustering (*see* **Note 2**).

Reproducibility is excellent (100%) both intra- and interlaboratory (***18,21***). The *spa* typing has been proved stable in vitro (***18***) and to a lesser extent in vivo (***13***). However, the longitudinal study of ten persistent infections of cystic fibrosis (CF) patients by a single *S. aureus* clone (as determined by PFGE) demonstrated occasional mutational events (deletions, point mutation, or duplication of *spa* repeats) in 10% of the sequential isolates studied (***22***). This finding could also be explained by the existence of closely related co-colonizing isolates, as described later by the same team (***23***), and cannot be generalized given the peculiarity of the ecological niche and selective pressure present in the airways of CF patients.

1.4.2. Epidemiological Concordance and Discriminatory Power

The use of *spa* typing for outbreak investigation was validated by Shopsin et al. (***18***) on a well-documented strain collection containing 29 isolates belonging to four distinct outbreaks. Discriminatory power of *spa* typing, evaluated on several large strain collections, was found to be similar to PFGE, with a Simpson Index of diversity ranging from 0.97 to 0.98 (***4,20,24***).

1.4.3. Concordance with Gold Standard Methods and Phylogenetic Inference

Besides a high discriminatory power, *spa* typing is usually in good concordance with PFGE (using the Tenover criteria (***25***)), either at the type level (from 96% to 98%) (***20,24***) or between clusters (93%, using BURP (based upon repeat patterns) algorithm; *see* **Subheading 3.4.2.)** (***4,24,26***). Furthermore, concordance with MLST and BURST (based upon related sequences) clustering also proved to be very high (97% to 99%) (***4,24***).

This ability of *spa* typing to combine a high discriminatory power with a high concordance with MLST as a single-locus marker resides in its repeat composition and organization. Point mutations (occurring at a low rate and subject to purifying selection) permit a reliable lineage assignment, while additions or deletions of repeats (fast-occurring) index intralineage variations, enabling the use of *spa* typing for both long- and short-term epidemiology (***19***).

However, when assuming that an isolate belongs to an MLST lineage based on its *spa* type, one should be aware of possible misclassification problems, as identified for a few lineages by several authors (***4,24,27,28***). Indeed, strains belonging to distant MLST CCs can present identical or similar *spa* profiles and cluster (using the BURP algorithm) together in a unique *spa* group. This still unexplained phenomenon could possibly be in certain cases due to recombination events involving the *spa* locus (***27***). Other cases can be caused by large chromosomal replacement encompassing the *spa* gene, such as described by Robinson and Enright for ST239 and ST34 (***28***).

2. Materials

The materials listed are only suggestions as many excellent alternatives exist.

2.1. DNA Extraction

1. Columbia agar plates with 5% sheep blood (Biomérieux, Marcy l'Etoile, France).
2. Lysostaphin solution (1 mg/mL) (AMBI Products LLC, New York).
3. Proteinase K solution (1 mg/mL) (Sigma).
4. DNase (deoxyribonuclease)-free water.
5. Tris-HCl, 0.1*M*, pH 8.0 solution.
6. Water baths or heater blocks (37, 60, 100°C).
7. Vortex.

2.2. Polymerase Chain Reaction (PCR)

1. AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA).
2. Deoxynucleotide 5′-triphosphates (dNTPs; Promega, Madison, WI).
3. High-performance liquid chromatographic-cleaned primers (spa-1113f, spa-1514r; for complete sequence, *see* **Subheading 3.2.**) (MWG-Biotech, Ebersberg, Germany).
4. PCR buffer II (Applied Biosystems).

5. End-point thermocycler: GeneAmp PCR System 9700 (Applied Biosystems).
6. Elution spin column: Kit Quantum prep PCR Kleen Spin Columns (Bio-Rad).

2.3. DNA Sequencing

1. ABI Prism BigDye Terminator V3.1 Sequencing Kit (Applied Biosystems).
2. ABI prism 4100 sequencing machine (Applied Biosystems).
3. Microplates: MultiScreen HV, clear plate 45UL (Millipore, Billerica, MA).
4. Sephadex G 50 (Amersham Biosciences, Freiburg, Germany).
5. Microplate centrifuge, up to 2100 g, rotor 11123 (Sigma).

2.4. Data Analysis

1. StaphType software (Ridom GmbH, Würzburg, Germany) or Bionumerics (Applied Maths, Ghent, Belgium).
2. Internet connection.

3. Methods

3.1. DNA Extraction

Multiple extraction protocols ranging from a simple boiling step (*29*) to commercial tissue or blood extraction kits (*27*) or glass bead mechanical lysis have been described to be suitable for *spa* typing (*see* **Note 3**). A rapid extraction protocol can be used as follows:

1. Suspend one colony of *S. aureus* cultured for 24 h on Columbia agar with 5% sheep blood in 45 µL of DNase-free water and 5 µL of lysostaphin (1 mg/mL), vortex, and incubate for 10 min at 37°C.
2. Add 45µL of DNase-free water, 5 µL of proteinase K 2 mg/mL and 150 µL of Tris-HCl 0.1*M,* pH 8.0; vortex and incubate 10 min at 60°C and then 5 min at 100°C.
3. Of this lysate, 5 µL is used as the DNA template in the PCR reaction.

3.2. DNA Amplification

Several pairs of primers have been described, numbered from the forward strand of *S. aureus* DNA (GenBank accession no. J01786), for example:

spa-1113f [1092–1113] (5′-TAA AGA CGA TCC TTC GGT GAG C) and spa-1514r [1534–1514] (5′-CAG CAG TAG TGC CGT TTG CT-3′) (*30*).

spa-1095f [1095–1113] (5′- AGA CGA TCC TTC GGT GAG C) and spa-1517r [1517–1496] (5′-GCT TTT GCA ATG TCA TTT ACT G-3′) (*17,18*).

The DNA amplification protocol (recommended by the Ridom software supplier, www.ridom.de) is the following:

1. Add genomic staphylococcal DNA in a PCR mixture to achieve 50 µL of final volume containing 1.25 units of *Taq* polymerase, 1.5 m*M* $MgCl_2$, 200 µ*M* dNTPs, 0.2 µ*M* of each primer (spa-1113f, spa-1514r) and 5 µL of 10X PCR buffer.
2. Cycling conditions consist of an initial denaturation step of 5 min at 80°C, followed by 35 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 60°C, 90 s of extension at 72°C, and a final extension step of 10 min at 72°C (*see* **Notes 4–6**).
3. The PCR product is then purified, either by an enzymatic method or by elution spin column, and can be stored at 4°C.

3.3. DNA Sequencing

1. Use the following reaction mix: 20 to 30 ng of amplified and purified DNA in a final reaction volume of 10 µL containing 2 µL of premix and 1 µL of buffer from the kit and 0.5 m*M* of each primer (use the same primers as for the amplification step).
2. Amplification parameters are the following: An initial denaturation step of 2 min at 96°C, followed by 25 cycles of 30 s of denaturation at 96°C, 15 s of annealing at 50°C, and 60 s of extension at 60°C.
3. The products are then purified and concentrated prior to sequencing, either by ethanol precipitation or by commercial elution spin columns (Dye-ex, Quiagen, Hilden, Germany) or microplates loaded with Sephadex G 50.

3.4. Data Interpretation

3.4.1. spa *Type Assignment*

The VNTR structure of the *spa* locus makes the traditional sequence alignment (using the substitutions, insertions, and deletions [indel] of a single-position model) improper to accurately identify *spa* repeat units and assess how these units are organized. Several programs, both "in-house" and commercial, have been described and can be successfully used for this purpose (***17,18,31***). Among those, the StaphType software (Ridom) is the most widely used. Other bioinformatic tools, such as Bionumerics (Applied Maths) also allow *spa* analysis and *spa* type designation using the Ridom nomenclature. Submissions from the Bionumerics software to the *spa* server will be possible in the near future via the online SeqNet gatekeeper interface (www.seqnet.org; *see* **Subheading 3.4.3.**). Both software include internal quality control systems that ensure that extrapolation of repeat and repeat organization are made on sequence data (chromatograms) of sufficient quality (*see* **Note 7**).

3.4.2. *Grouping of Related* spa *Types*

Until recently, the only way to cluster or classify *spa* types was to visually estimate the similarity of their repeat profiles. This method, although feasible with limited size strain collections and found to concordantly classify strains as compared with MLST

results (***20,29,32,33***), is difficult to apply to large collections. The BURP algorithm is an automated algorithm—implemented in the StaphType software—that can cluster (*spa*-CC) *spa* types (***17,34***). Repeat duplication and excision are taken into account (in addition to single-position substitution and indel events) when the relatedness of different *spa* types is calculated. A "cost" accounting for the "steps" of evolution between each examined pair of *spa* types is calculated, whereas the algorithm tries to minimize these steps (parsimony assumption) (***34***). BURP offers two user-defined parameters that influence clustering: exclusion of *spa* types that are shorter than *x* repeats and the maximum number of costs *y* for clustering *spa* types into the same group. Using these parameters, short *spa* types (presenting limited evolutionary information) can be excluded from further analysis (*see* **Note 2**), and maximum costs can be adapted to the size of the strain collection studied and the question asked in terms of space and time evolutionary scale (small versus large outbreak investigation versus long-term epidemiological surveillance studies) (*see* **Note 8**).

3.4.3. The StaphType Software

The StaphType software combines three modules: a sequence editor, a database, and a report generator. For each *spa* sequence downloaded by the software, epidemiological information concerning the isolate typed can be recorded in the database module.

3.4.3.1. Sequence Analysis and spa *Type Designation*

The sequence analysis starts with the download of both forward and reverse sequences files (FASTA format or preferably ABI and SCF chromatograms). The software searches then automatically for the 5′ and 3′ signature sequences (conserved flanking regions), constructs a consensus sequence, and detects the *spa* repeats succession (***17***). In case of already known *spa* repeat succession, the *spa* type designation is automatically downloaded from the *spa* server Web site. New *spa* repeats and *spa* types detected by one laboratory using the StaphType software are automatically given a preliminary name in the local database (e.g., txAA or txAB). The laboratory typing data can then be synchronized via the Internet; the new sequences are then matched automatically with *spa* types found by other participants. If the repeat succession is revealed as new, a new type number is assigned for all future detection of this *spa* repeat profile, ensuring a continuously updated common nomenclature. Type numbers are assigned by the order of submission; no relatedness can be deduced from the closeness of two t-numbers. If the repeat succession has already been described and synchronized by another laboratory, the preliminary name in the local database of the inquiring laboratory is automatically changed to the preexisting denomination.

3.4.3.2. *Automated Quality Control of* spa *Typing Data*

As the *spa* server receives more than 1,000 submissions per month, a major goal is the maintenance of excellence of the data

quality. Therefore, the curator of the SeqNet.org database (*see* **Subheading 3.4.5.**) has set up rules for procedure and internal and external quality control schemes:

1. An internal quality control system is integrated in the StaphType software: To each downloaded sequence is attached a quality index, which corresponds to a sequence error probability. The *spa* typing sequences with low reliability cannot be synchronized via the server and are rejected by the SeqNet curator (*see* **Note 9**).
2. The external quality control consists of the performance of a certification for all new SeqNet.org members and a regular proficiency test, based on known ring trials. This external quality certificate is sent out to the laboratory when capacities for high-quality sequence typing has been established. During the certification process, the curators assist the new SeqNet.org aspirants in the development of sequence capacity, often accompanied by a 3-d stay at the sequencing facilities of the coordinators or participating in one of the hands-on laboratory workshops.

3.4.3.3. Data Ownership

Data ownership on the *spa* server is ruled by the SeqNet.org initiative, which curates the data for all submitters. It is important to mention that all data on the *spa* server are strictly incrementally synchronized. This means that all synchronized data, after passing quality control and assignment of the *spa* type, are stored with a single laboratory identifier. Every submitter using direct submission can choose which epidemiological data should be shared on the Web site (s*ee* **Note 10**). International study groups or regional and national networks can opt for not making visible their data submission on the public home page as long as wished by the interested group. In this way, intellectual data property of each single submitter is protected.

3.4.4. The Central spa Server

The Harmsen et al. nomenclature for the designation of *spa* types has been made universally accessible by establishing the central *spa* server. It allows the automated quality control of submitted sequence data, and the central synchronization rends the submitted data publicly available on the online Web site (www.SpaServer.ridom.de). Users of other *spa* analyzing software tools than StaphType are able to synchronize with the *spa* server via an online uploading interface while fulfilling all given quality criteria checked by the SeqNet.org curators. Until now, agreements between SeqNet.org and two developers of *spa* analyzing software (Ridom at www.ridom.de and Applied Maths at www.applied-maths.com) have been achieved. SeqNet.org will serve as gatekeeper for quality for the synchronization of *spa* sequences from submitters using one of the *spa* analyzing tools.

3.4.5. The SeqNet.org spa Typing Network

The central *spa* server (which has been developed by Ridom) is curated by the SeqNet.org initiative (***35***) on behalf of all users. SeqNet.org currently is an initiative of 45 laboratories from 25 European countries (1 laboratory from Lebanon) founded in 2004 at the University of Münster in Germany (http://www.SeqNet.org). Its main objective is to establish a European network of excellence for sequence-based typing of microbial pathogens, having its main focus on *S. aureus*. SeqNet.org comprises a large number of national reference laboratories as well as university and some veterinary laboratories. The principle goal of SeqNet.org is to create unambiguous, electronic, portable, easily comparable typing data of excellent quality for local infection control and national and European surveillance of sentinel microorganisms, such as MRSA.

Currently, parallel to the SeqNet.org laboratories, more than 140 other submitting laboratories have synchronized their *spa* types with the database. Although, the *spa* database in its current form essentially is used as a *spa* type dictionary, ensuring a common nomenclature, providing molecular typing data in real time, and maintaining typing data quality, its data entries on frequencies of *spa* types and country of submission can already provide valuable information regarding geographical dissemination and occurrence of the *spa* types by country (***36***).

Furthermore, the *spa* server can be used by regional, national, or international public health or research networks to filter *spa* data from the network's participating laboratories, hospitals, and medical practices. Important examples are the Dutch-German cross-border networks EUREGIO MRSA-net Twente/Münsterland (***37,38***) and the MRSA network of the EUREGIO Maas-Rhein (***39***) In both cases, *spa* typing ensures not only the intrahospital but also the cross-border comparability and Euregional data ownership of the typing data.

4. Notes

1. A translating tool from one nomenclature to the other can be downloaded from the *spa* server Web site (www. SpaServer.ridom.de).
2. Isolates presenting short *spa* profile, although technically typable, should be excluded from clustering analysis. Five has been proposed (***34***) as the minimum number of repeats necessary to infer relatedness.
3. An initial "staphylococcal-specific" lysis step using lysostaphin (24,27) is however recommended to ensure sufficient bacterial lysis.

4. Several other amplification protocols have been described and proved to be efficient (***18,40***).
5. Visualization of the amplified DNA by conventional electrophoresis in 1 or 2% agarose gel is recommended prior to the sequencing step. The average amplified product size should be between 300 and 600 bp but varies following the number of *spa* repeats.
6. For isolates that are nontypable using the primers cited, SeqNet.org recommends using the following primers (A. Mellmann, personal communication 2007).
   (a) spa-239f (5′-ACTAGGTGTAGGTATTGCATCTGT-3′)
   (b) spa-1717r (5′-TCCAGCTAATAACGCTGCACCTAA-3′)
   (c) spa-1084f (5′-ACAACGTAACGGCTTCATCC-3′)
   (d) spa-1618r (5′-TTAGCATCTGCATGGTTTGC-3′)

   However, 1 of 1,000 isolates remains nontypable. The reason might be that some *S. aureus* isolates present large deletions in the *spa* gene that can affect the primer binding sites.
7. Manual editing of *spa* sequence data should be avoided as much as possible because it can easily lead to misidentification of repeats and subsequently to the attribution of an incorrect *spa* type.
8. This parameter is by default set to four by the Ridom software. However, this was calibrated to suit long-term evolution characterization (i.e., maximal concordance with MLST data) (***34***). For small outbreak investigation, this parameter can be lowered.
9. If the 5′/3′ signatures are found, the repeats are correct, and the sequence is traced correctly, the reliability value given is 100 (good). If 5′/3′ signatures are found, the repeat succession contains no low-quality basis, and there is a consensus of traces, then the reliability value for quality given is 110 (very good) (***12***). Last, if the criteria for 110 are fulfilled and there are fewer than five editing steps of the sequence, the reliability value for quality given is 120 (excellent). In the case that the 5′/3′ signatures are not correctly found, signature positions are shifted, or base quality is low, the reliability values are between 90 and 40 (sufficient) or between 30 and 0 (poor) (***33***). Each *spa* typing sequence with a reliability value lower than 100 cannot be synchronized via the server and is rejected by the SeqNet curators.
10. The submitter is also able to withdraw his or her data at any time by resynchronizing with the server and indicating the deletion of its submission. In such case, only the *spa* type and the information on the sequence quality will remain on the server.

References

1. Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med.* **339**, 520–532.
2. Jevons, M. P. (1961). "Celbenin"-resistant Staphylococci. *BMJ* **1**, 124–125.
3. Aires, d. S., and de Lencastre, H. (2004). Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. *FEMS Immunol. Med. Microbiol.* **40**, 101–111.
4. Strommenger, B., Kettlitz, C., Weniger, T., Harmsen, D., Friedrich, A. W., and Witte, W. (2006). Assignment of *Staphylococcus* isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. *J. Clin. Microbiol.* **44**, 2533–2540.
5. Tenover, F. C., Arbeit, R., Archer, G., Biddle, J., Byrne, S., Goering, R., et al. (1994). Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J. Clin. Microbiol.* **32**, 407–415.
6. Murchan, S., Kaufmann, M. E., Deplano, A., De Ryck, R., Struelens, M., Zinn, C. E., et al. (2003). Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus:* a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J. Clin. Microbiol.* **41**, 1574–1585.
7. Deplano, A., Schuermans, A., Van Eldere, J., Witte, W., Meugnier, H., Etienne, J., et al. (2000). Multicenter evaluation of epidemiological typing of methicillin-resistant *Staphylococcus aureus* strains by repetitive-element PCR analysis. The European Study Group on Epidemiological Markers of the ESCMID. *J. Clin. Microbiol.* **38**, 3527–3533.
8. Cookson, B. D., Aparicio, P., Deplano, A., Struelens, M., Goering, R., and Marples, R. (1996). Inter-centre comparison of pulsed-field gel electrophoresis for the typing of methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol.* **44**, 179–184.
9. Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., and Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* **38**, 1008–1015.
10. Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., and Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci. U S A* **99**, 7687–7692.
11. Peacock, S. J., de Silva, G. D., Justice, A., Cowland, A., Moore, C. E., Winearls, C. G., et al. (2002). Comparison of multilocus sequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* isolates in a microepidemiological setting. *J. Clin. Microbiol.* **40**, 3764–3770.
12. Frenay, H. M., Theelen, J. P., Schouls, L. M., Vandenbroucke-Grauls, C. M., Verhoef, J., van Leeuwen, W. J., et al. (1994). Discrimination of epidemic and nonepidemic methicillin-resistant *Staphylococcus aureus* strains on the basis of protein A gene polymorphism. *J. Clin. Microbiol.* **32**, 846–847.
13. Frenay, H. M., Bunschoten, A. E., Schouls, L. M., van Leeuwen, W. J., Vandenbroucke-Grauls, C. M., Verhoef, J., et al. (1996). Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**, 60–64.
14. Guss, B., Uhlen, M., Nilsson, B., Lindberg, M., Sjoquist, J., and Sjodahl, J. (1984). Region X, the cell-wall-attachment part of staphylococcal protein A. *Eur. J. Biochem.* **138**, 413–420.
15. van Belkum, A. (1999). The role of short sequence repeats in epidemiologic typing. *Curr. Opin. Microbiol.* **2**, 306–311.
16. van Belkum, A. (2007). Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). *FEMS Immunol. Med. Microbiol.* **49**, 22–27.
17. Harmsen, D., Claus, H., Witte, W., Rothganger, J., Claus, H., Turnwald, D., et al. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J. Clin. Microbiol.* **41**, 5442–5448.
18. Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., et al. (1999). Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **37**, 3556–3563.
19. van Belkum, A., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K., et al. (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin. Microbiol. Infect.* **13** (Suppl. 3), 1–46.
20. Koreen, L., Ramaswamy, S. V., Graviss, E. A., Naidich, S., Musser, J. M., and Kreiswirth, B. N. (2004). *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *J. Clin. Microbiol.* **42**, 792–799.

21. Aires-de-Sousa, M., Boye, K., de Lencastre, H., Deplano, A., Enright, M. C., Etienne, J., et al. (2006). High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *J. Clin. Microbiol.* **44**, 619–621.
22. Kahl, B. C., Mellmann, A., Deiwick, S., Peters, G., and Harmsen, D. (2005). Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus. J. Clin. Microbiol.* **43**, 502–505.
23. Goerke, C., Gressinger, M., Endler, K., Breitkopf, C., Wardecki, K., Stern, M., et al. (2007). High phenotypic diversity in infecting but not in colonizing *Staphylococcus aureus* populations. *Environ. Microbiol.* **9**, 3134–3142.
24. Hallin, M., Deplano, A., Denis, O., de Mendonca, R., De Ryck, R., and Struelens, M. J. (2007). Validation of pulsed-field gel electrophoresis and *spa* typing for long-term, nationwide epidemiological surveillance studies of *Staphylococcus aureus* infections. *J. Clin. Microbiol.* **45**, 127–133.
25. Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., et al. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**, 2233–2239.
26. Faria, N. A., Carrico, J. A., Oliveira, D. C., Ramirez, M., and de Lencastre, H. (2008). Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **46**, 136–144.
27. Strommenger, B., Braulke, C., Heuck, D., Schmidt, C., Pasemann, B., Nubel, U., et al. (2008). *spa* typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing *J. Clin. Microbiol.* **46**, 574–581.
28. Robinson, D. A., and Enright, M. C. (2004). Evolution of *Staphylococcus aureus* by large chromosomal replacements*J. Bacteriol.* **186**, 1060–1064.
29. Ruppitsch, W., Indra, A., Stoger, A., Mayer, B., Stadlbauer, S., Wewalka, G., et al. (2006). Classifying *spa* types in complexes improves interpretation of typing results for methicillin-resistant *Staphylococcus aureus. J. Clin. Microbiol.* **44**, 2442–2448.
30. Mellmann, A., Friedrich, A. W., Rosenkotter, N., Rothganger, J., Karch, H., Reintjes, R., et al. (2006). Automated DNA sequence-based early warning system for the detection of methicillin-resistant *Staphylococcus aureus* outbreaks. *PLoS Med.* **3**, e33.
31. Oliveira, D. C., Crisostomo, I., Santos-Sanches, I., Major, P., Alves, C. R., Aires-de-Sousa, M., et al. (2001). Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus. J. Clin. Microbiol.* **39**, 574–580.
32. Feil, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., et al. (2003). How clonal is *Staphylococcus aureus*? *J. Bacteriol.* **185**, 3307–3316.
33. Robinson, D. A., and Enright, M. C. (2003). Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus. Antimicrob. Agents Chemother.* **47**, 3926–3934.
34. Mellmann, A., Weniger, T., Berssenbrugge, C., Rothganger, J., Sammeth, M., Stoye, J., et al. (2007). Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on *spa* polymorphisms. *BMC Microbiol.* **7**, 98.
35. Friedrich, A. W., Witte, W., Harmsen, D., de Lencastre, H., Hryniewicz, W., Scheres, J., et al. (2006). SeqNet.org: a European laboratory network for sequence-based typing of microbial pathogens. *Euro Surveill.* **11**, E060112.
36. von Eiff, C., Maas, D., Sander, G., Friedrich, A. W., Peters, G., and Becker, K. (2008). Microbiological evaluation of a new growth-based approach for rapid detection of methicillin-resistant *Staphylococcus aureus. J. Antimicrob. Chemother.* **61**, 1277–1280.
37. Friedrich, A., Daniels-Haardt, I., Gemert-Pijnen, J., Hendrix, M., von Eiff, C., Becker, K., et al. (2007). A regional network for the prevention and control of infections due to MRSA: EUREGIO MRSA-net Twente/Münsterland. *Epidemiol. Bull.* **33**, 307–311.
38. Daniels-Haardt, I., Verhoeven, F., Mellmann, A., Hendrix, M. G., Gemert-Pijnen, J. E., and Friedrich, A. W. (2006). [EUREGIO-projekt MRSA-net Twente/Munsterland. Creation of a regional network to combat MRSA]. *Gesundheitswesen* **68**, 674–678.
39. Deurenberg, R. H., Vink, C., Oudhuis, G. J., Mooij, J. E., Driessen, C., Coppens, G., et al. (2005). Different clonal complexes of methicillin-resistant *Staphylococcus aureus* are disseminated in the Euregio Meuse-Rhine region. *Antimicrob. Agents Chemother.* **49**, 4263–4271.
40. Oliveira, D. C., Crisostomo, I., Santos-Sanches, I., Major, P., Alves, C. R., Aires-de-Sousa, M., et al. (2001). Comparison of DNA

sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* **39**, 574–580.

41. Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., and Lindberg, M. (1984). Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J. Biol. Chem.* **259**, 1695–1702.