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High Interlaboratory Reproducibility of DNA Sequence-Based Typing of Bacteria in a Multicenter Study

M. Aires-de-Sousa,[1] K. Boye,[2] H. de Lencastre,[1] A. Deplano,[3] M. C. Enright,[4] J. Etienne,[5] A. Friedrich,[6] D. Harmsen,[7]* A. Holmes,[8] X. W. Huijsdens,[9] A. M. Kearns,[8] A. Mellmann,[6] H. Meugnier,[5] J. K. Rasheed,[10] E. Spalburg,[9] B. Strommenger,[11] M. J. Struelens,[3] F. C. Tenover,[10] J. Thomas,[4] U. Vogel,[12] H. Westh,[2] J. Xu,[13] and W. Witte[11]

Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Oeiras, Portugal[1]; Department of Clinical Microbiology, Hvidovre Hospital, Hvidovre, Denmark[2]; Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium[3]; Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom[4]; Laboratoire Central de Microbiologie, Hôpital Edouard Herriot, Lyon, France[5]; Institut für Hygiene, University Hospital Münster, Münster, Germany[6]; Department of Periodontology, University Hospital Münster, Münster, Germany[7]; Center for Infections, Health Protection Agency, London, United Kingdom[8]; Diagnostic Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands[9]; Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, Georgia[10]; Robert-Koch-Institut, Wernigerode, Germany[11]; Institut für Hygiene und Microbiologie, University Hospital Würzburg, Würzburg, Germany[12]; and State Key Laboratory of Infectious Diseases Prevention and Control, National Institute for Communicable Disease Prevention and Control, Beijing, People's Republic of China[13]

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Current DNA amplification-based typing methods for bacterial pathogens often lack interlaboratory reproducibility. In this international study, DNA sequence-based typing of the *Staphylococcus aureus* protein A gene (*spa*, 110 to 422 bp) showed 100% intra- and interlaboratory reproducibility without extensive harmonization of protocols for 30 blind-coded *S. aureus* DNA samples sent to 10 laboratories. Specialized software for automated sequence analysis ensured a common typing nomenclature.

Typing of pathogens is essential to detect outbreaks and to forecast future trends. For *Staphylococcus aureus*, a major healthcare-associated pathogen worldwide, pulsed-field gel electrophoresis (PFGE) is currently regarded as the molecular typing "gold standard" (11). Significant efforts have been made in the past to harmonize protocols of PFGE and to establish a standardized nomenclature. However, these projects proved only partially successful when judged by reproducibility, speed, and costs of analysis (11, 16, 17). To improve the speed of typing, DNA sequence-based approaches, such as multilocus sequence typing (MLST), are coming to be more frequently used for *S. aureus* (4). However, MLST is not suitable for routine surveillance of methicillin-resistant *S. aureus* (MRSA) because of higher costs and lower discriminatory power compared to PFGE. Frenay et al. developed a single-locus sequence typing method for *S. aureus* using the sequences of polymorphic region X of the *S. aureus* protein A (*spa*) gene (5). Subsequently, it was shown by calculation of Simpson's index of diversity that *spa* typing is nearly as discriminatory as PFGE (7, 13). To date, no study has investigated the interlaboratory comparability of DNA sequence-based genetic typing methods. Therefore, we evaluated the reproducibility of *spa* sequencing in a multicenter study using specialized software for sequence analysis.

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To examine interlaboratory comparability, 30 blind-coded *S. aureus* DNA samples (Table 1) were shipped worldwide by mail to 11 laboratories with personnel experienced in DNA sequencing (Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Oeiras, Portugal; Hvidovre Hospital, Hvidovre, Denmark; Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium; Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom; Laboratoire Central de Microbiologie, Hôpital Edouard Herriot, Lyon, France; Institut für Hygiene, University Hospital Münster, Münster, Germany; Center for Infections, Health Protection Agency, London, United Kingdom; Diagnostic Laboratory of Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, GA; Institut für Hygiene und Microbiologie, University Hospital Würzburg, Würzburg, Germany; State Key Laboratory for Infectious Diseases Prevention and Control, National Institute for Communicable Disease Prevention and Control, Beijing, People's Republic of China). Isolates 2, 3, 9, 12, and 28, representing different clonal lineages of MRSA, had been included in an international study on the evolutionary origin of MRSA (3). The remaining isolates used in this study originated from the strain collection of the German Reference Center for Staphylococci and were unrelated with respect to time and place of infection. Of the 30 DNA samples, one (no. 2) was supplied in four replicates (11, 15, 17, and 25) to test intralabo-

* Corresponding author. Mailing address: Department of Periodontology, University Hospital Münster, Waldeyerstrasse 30, D-48149 Münster, Germany. Phone: 49-251-9802875. Fax: 49-251-9802868. E-mail: dharmsen@uni-muenster.de.

TABLE 1. Strains used for interlaboratory comparison of *spa* typing

No.[a]	Strain[b]	*spa* type[c] (sequence length in bp)	MLST ST[d]	Comment(s)[e]
1	PS80	t021 (254)	ST30	MSSA, "classical" isolate of *lukS-lukF*$^+$ nosocomial *S. aureus* of MLST ST 30 (15)
2	1678/96	t032 (422)	ST22	MRSA, SCC*mec* IVc, prototype of ST22 MRSA in Europe, "Barnim MRSA"
3	1976/98	t018 (302)	ST36	MRSA, SCC*mec* III, prototype of ST36 MRSA
4	1451/97	t308 (275)	ST121	MSSA, prototype of *lukS-lukF*$^+$, *S. aureus* from furunculosis
5	2878/02	t285 (299)	ST121	MSSA, *lukS-lukF*$^+$, *S. aureus* from furunculosis
6	2800/00	t159 (251)	ST121	MSSA, superficial infection of skin (*eta*$^+$ *etb*$^+$)
7	1488/00	t269 (155)	ST121	MSSA, exfoliative dermatitis (*eta*$^+$ *etb*$^+$)
8	544/95	t159 (251)	ST427	MSSA, *lukS-lukF*$^+$ from tropical pyomyositis, SLV of ST121
9	1000/93	t009 (350)	ST254	MRSA, SCC*mec* IV
10	2280/03	t008 (278)	ST254	MRSA, SCC*mec* IV
11	1678/96	t032 (422)	ST22	Replicate of strain 2 for intralaboratory reproducibility testing
12	134/93	t051 (302)	ST247	MRSA, SCC*mec* I, "Iberian"/"Northern German MRSA"
13	406/98	t051 (302)	ST247	MRSA, SCC*mec* I, h-GISA in western and central Europe
14	2404/02	t044 (206)	ST80	MRSA, SCC*mec* IV, *lukS-lukF*$^+$, cMRSA widely disseminated in Europe (15)
15	1678/96	t032 (422)	ST22	Replicate of strain 2 for intralaboratory reproducibility testing
16	2773/03	t175 (278)	ST1	MRSA, SCC*mec* IVa, *lukS-lukF*$^+$, EMRSA, widely disseminated in United States (15)
17	1678/96	t032 (422)	ST22	Replicate of strain 2 for intralaboratory reproducibility testing
18	1293/00	t032 (422)	ST22	MRSA, SCC*mec* IVc, subclone of ST22 prototype according to SmaI PFGE pattern
19	608/04	t310 (278)	ST22	MRSA, SCC*mec* IV, *lukS-lukF*$^+$
20	609/04	t309 (254)	ST22	MRSA, SCC*mec* IV, *lukS-lukF*$^+$
21	1155/98-2	t001 (257)	ST228	MRSA, SCC*mec* I, prototype of ST228 MRSA, "Southern German MRSA"
22	653/99	t001 (257)	ST228	MRSA, SCC*mec* I, subclone of ST228 MRSA according to SmaI PFGE pattern
23	2106/03	t002 (254)	ST5	MRSA, SCC*mec* II, "Rhine Hesse MRSA"
24	97S101	t045 (206)	ST5	MRSA, SCC*mec* IV
25	1678/96	t032 (422)	ST22	Replicate of strain 2 for intralaboratory reproducibility testing
26	2121/03	t002 (254)	ST5	MRSA, unknown SCC*mec* with unknown *ccr* complex
27	PS95	t065 (254)	ST45	MSSA, widely disseminated putative ancestor of ST45 MRSA
28	1150/93	t004 (254)	ST45	MRSA, SCC*mec* IV, "Berlin MRSA"
29	2756/02	t038 (254)	ST45	MRSA, SCC*mec* IV, subclone of "Berlin MRSA" in North Rhine-Westphalia
30	2712/02	t026 (110)	ST47	MRSA, SCC*mec* IV, SLV of ST45

[a] Blind-coded number used for all participants.
[b] Original strain designation.
[c] *spa* type nomenclature in accordance with http://www.spaServer.ridom.de.
[d] ST, sequence type; nomenclature in accordance with http://saureus.mlst.net.
[e] *lukS-lukF*$^+$, two-component leukocidin positive, encodes Panton-Valentine leukocidin; SCC*mec*, staphylococcal chromosomal cassette encodes methicillin resistance; MSSA, methicillin-susceptible *S. aureus*; *eta*$^+$ *etb*$^+$: exfoliative toxin gene A and B positive; SLV, MLST single-locus variant; h-GISA, *S. aureus* with heterogeneous resistance to glycopeptides; cMRSA, community MRSA; EMRSA, epidemic MRSA clone; *ccr* complex, cassette chromosome recombinase complex.

ratory reproducibility (Table 1). Investigators sequenced the *spa* gene by using in-house sequencing platforms and analyzed the resulting forward and reverse sequence chromatograms (ABI, SCF, or FASTA file format) with the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). The software attaches to each called base a quality value that corresponds to a sequence error probability. Taking the quality values into consideration, the software constructs a consensus sequence, automatically detects the *spa* repeats, and assigns a *spa* type. In at least 95% of all cases, no further manual editing is necessary. For the remaining sequences, a graphic user interface allows the user to edit sequences manually by means of an integrated expert system (6). A *spa* type is deduced from the order of specific repeats. Exchange of a single base results in a different *spa* type.

A *spa* PCR and sequencing protocol was made available. The protocol suggested using PuRe *Taq* Ready-To-Go PCR Beads (Amersham Biosciences, Freiburg, Germany) in a total volume of 25 μl containing LiChrosolv water (Merck, Darmstadt, Germany), DNA, and 10 pmol each of high-performance liquid chromatography-cleaned primers spa-1113f (5′-TAA AGA CGA TCC TTC GGT GAG C-3′) and spa-1514r (5′-CAG CAG TAG TGC CGT TTG CTT-3′). Thermal cycling reaction conditions consisted of initial denaturation (5 min at 80°C), followed by 35 cycles of denaturation (45 s at 94°C), annealing (45 s at 60°C), and extension (90 s at 72°C), and finishing with a single extension (10 min at 72°C). Twenty microliters of each PCR product was purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced with the ABI Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany). The sequencing reaction mixture contained LiChrosolv, 0.5 μl of premix, and 2 μl of ABI 5x Sequencing Buffer from the kit; 10 pmol of sequencing primer; and 2 μl of cleaned PCR product in a total volume of 10 μl. The amplification primers were used for sequencing by 25 cycles of denaturation (10 s at 96°C) and extension (4 min at 60°C) at a thermal ramping rate of 1°C/s. The sequencing products were purified with a DyeEx 2.0 Spin Kit (QIAGEN) and then prepared for running on an ABI 3100 Avant Genetic Analyzer in accordance with the manufacturer's (Applied Biosystems) instructions. Apart from the primers, all participants in this study were free to use their routine procedures.

One laboratory did not receive the DNA samples; the remaining 10 laboratories were able to determine and analyze the sequences of the 30 DNA samples with the Ridom StaphType software. Harmonization and adherence to the recommended protocol, which are typically requirements in most typing studies (11, 15, 17), were not needed. Each laboratory determined

8,691 bp (range, 110 to 422 bp per strain), and all participants reported exactly the same *spa* type for each of the isolates analyzed. Therefore, the intra- and interlaboratory reproducibility of the sequencing results was 100% each. Sending DNA samples instead of bacterial isolates facilitated the exchange of specimens. Our results prove the excellent reproducibility of sequence-based typing methods due to the unambiguous nature of sequence data. In addition, the use of the StaphType software ensured a uniform terminology by synchronization with an accompanying public website administering all new *spa* repeat and type codes (6). Only high-quality sequence data are automatically accepted by the server (http://www.SpaServer.ridom.de), and therefore no curator is needed for administration of the database or analyses of newly submitted sequences and strains, in contrast to other typing networks, e.g., MLST.net (http://www.mlst.net).

This study presents a global multicenter evaluation of DNA sequence-based typing in bacteriology. Due to its clinical importance and the availability of specialized software, *S. aureus* was chosen as the model organism for sequence-based typing. Numerous collaborative studies have shown limited intra- and interlaboratory reproducibility for PFGE (1, 10, 11, 17). Other molecular typing methods, e.g., repetitive-element PCR, also failed to achieve satisfying reproducibility (2). Only the reproducibility of DNA sequencing was the subject of this study, whereas the typeability, discriminatory power, and stability of *spa* sequences (14) have already been demonstrated elsewhere (5, 6, 8). As costs associated with DNA sequencing are decreasing, this methodology now is a viable alternative for molecular typing of pathogens and becomes feasible even for smaller laboratories (12). Its demonstrated portability and reproducibility offer the prospect of a future "molecular Esperanto" typing standard (9).

In conclusion, genotyping of bacterial isolates by DNA sequencing proved to be a highly reproducible and robust technique even without extensive harmonization of laboratory protocols. The use of specialized software for the analysis of sequencing data ensured a common typing nomenclature and thus greatly facilitated the exchange of typing data.

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